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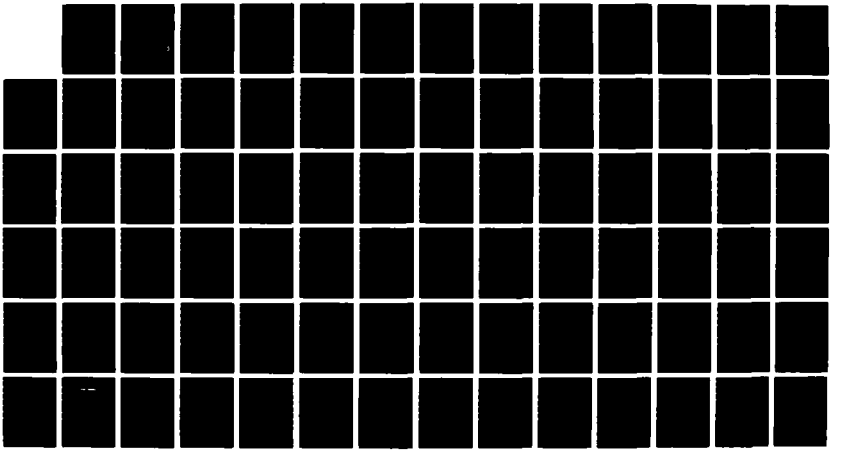

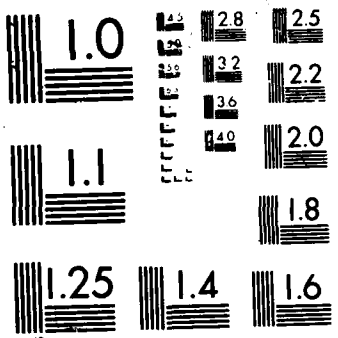
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Department of Physiology and Biophysics
University of Tennessee, Memphis
894 Union Avenue
Memphis, Tennessee 38163

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Previously, our research dealt with the somnogenic actions of factors isolated from rabbit brain and human urine. We identified these substances as muramyl peptides (MPs) and subsequently showed that a simple chemical analog to these MPs, N-acetylmuramyl-L-alanyl-D-isoglutamine (or MDP for muramyl dipeptide) was also somnogenic. Thus, at the start of the contract period, our evidence indicated that MPs have the capacity to enhance SWS and several other laboratories had confirmed and extended that finding. The broad goal of the USAMRDC supported research is to develop the information that is necessary for the evaluation of MPs as sedatives for military use. This year, five sets of experiments were performed with this goal in mind. Results from each follow.

1) The effects of MDP on rabbit rapid-eye movement sleep (REMS) and relationships between MDP-altered sleep and thermoregulation had not been investigated; thus, we did so. We found that: a) intracerebroventricular administration of MDP reduced REMS, b) a stereoisomer of MDP, MDP-DD, failed to affect sleep, c) acute elevation of ambient temperature

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(Abstract, continued)

enhanced rabbit slow-wave sleep (SWS) and, at that temperature, administration of MDP further enhanced SWS, and d) MDP enhanced SWS whether given during light or dark hours, although MDP-induced fever was greater during light hours. We conclude that MDP reduces REMS, that its effect on thermoregulatory processes depends on the time of injection and that the effects of thermoregulatory process on sleep are independent of the effects of MDP on sleep.

2) The interaction of MDP with two drugs of military interest, amphetamine and eserine, was examined. We found that MDP could reverse amphetamine-induced insomnia but that MDP failed to reverse eserine-induced wakefulness. We conclude that MDP does not act directly on thalamocortical cholinergic mechanisms of EEG phenomena. These results together with earlier evidence suggest that the somnogenic actions of MPs likely involve the midbrain.

3) Previously we had described several MP structure/somnogenic activity relationships. This year this knowledge was expanded; we found that O-acetylation of the 6-carbon of muramic acid enhanced somnogenic activity of an MP.

4) Many MPs are also immune response modifiers as well as pyrogenic and somnogenic. Thus, we measured the somnogenic effects of MDP in conjunction with a biochemical measure of the host defense response, plasma Cu. We found that central administration of MDP induced a dose-dependent rise in plasma Cu which was associated with MDP-induced sleep and fever. The effect on plasma Cu was due to a central mechanism since intravenous injection of the same doses of MDP that were used centrally failed to alter plasma Cu.

5) MPs can alter the production of cytokines such as interleukin-1 and tumor necrosis factor. Indeed, previously we postulated that the somnogenic actions of MPs may be mediated via interleukin-1. This year we showed that both recombinant DNA-derived interleukin-1 and tumor necrosis factor have the capacity to enhance SWS in rabbits.

The above results are discussed within the context of sleep-immune interactions.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Increased sleepiness is a common experience during either prolonged wakefulness (W) or infectious disease. A sleep-promoting substance, factor S, was first described as a constituent of cerebrospinal fluid (CSF) that increases in concentration during sleep deprivation (34). Later, substances with physical, chemical, and biological properties indistinguishable from those of factor S were isolated from brain and urine and identified as muramyl peptides (62). The most active component of the urinary material was completely characterized as N-acetylglucosaminyl-N-acetyl-1,6-anhydromuramyl-alanyl-glutamyl-diaminopimelyl-alanine (60). This substance and certain of its analogs induce sleep that mimics the sleep which follows sleep deprivation; i.e., they enhance slow-wave sleep (SWS) and electroencephalographic (EEG) slow wave amplitudes, whereas other aspects of sleep are left relatively undisturbed. Subsequently, we and others showed that a synthetic analog to factor S, muramyl dipeptide or MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine, is somnogenic in rabbits (63, 64, 57), rats (50, 51, 46, 80, 103), cats (63), and monkeys (121).

At the time the sleep-promoting materials from brain and urine were identified as muramyl peptides (MPs), it was recognized that they were closely related to the monomeric building blocks of bacterial cell wall peptidoglycan (63). Thus, it appeared that perhaps the molecular mechanisms which are responsible for the feelings of sleepiness that accompany sleep deprivation are identical to those associated with infectious disease. Certain MPs are also immune response modifiers (23, 24) and pyrogenic (55). These actions are thought to be mediated in part by the cytokines interleukin-1 (IL1), interferon$_{\alpha}$ (IFN), and tumor necrosis factor (TNF). These cytokines, which are

also CNS products (35, 72), were subsequently shown to be somnogenic, initially by us and later confirmed by others (66, 93, 107, 113, 120). It seemed, therefore, that MPs elicted their somnogenic actions via a step that involved enhanced cytokine production. However, it is well known that normal sleep is not accompanied by fever and may not be associated with activation of the immune response. Therefore, it was important to show that these activities could be separated from the somnogenic activities of MPs. Thus, coadministration of an antipyretic attenuates MP-induced febrile responses, but not sleep responses (63). In neonates, MPs induced enhanced sleep, but not fever (27, 28). In rats, low doses of MDP enhance sleep, but not body temperature (50, 51). Further, some MPs are pyrogenic, but not somnogenic (64). Similarly, some MPs are immunologically active, but not somnogenic (67).

Thus, at the start of the contract period, our evidence suggested that MPs have the capacity to enhance SWS. Several laboratories had confirmed this finding, and we and others provided evidence suggesting that the pyrogenic and immunologic action of MPs could be separated from their somnogenic actions. Because of these results, we thought it likely that, in the long run, new, more effective and safer somnogenic agents could be developed using MPs. Therefore, the broad objective of our studies reported here was the development of information needed to ascertain if MPs could in fact be developed into somnogenic agents. To provide this information, this year, the first year of our contract period, we began by focusing on five specific topics. In this report, these experiments are numbered 1-5 as follows: 1) We began by developing an extended description of what MDP does to rabbit sleep at different ambient temperatures at different times of the day. 2) Our second set of experiments dealt with the interaction of MDP with amphetamine and eserine.

3) We expanded our MP-structure-somnogenic activity knowledge by testing several new MPs derived from bacteria. 4) We also measured the effects of MPs in conjunction with a biochemical measure of the host defense response, i.e., change in plasma Cu. 5) Finally, we determined if recombinant DNA-derived monokine products were somnogenic.

In parallel with the above-mentioned work, we have also carried out research that is directly related to the USAMRDC-supported research. Because this work is chiefly supported by other agencies, NIH and ONR, it is not presented here; some references are 26-28, 57-59, 61, and 64.

METHODS

Materials

MDP and MDP-DD were from Institute Pasteur Productions, Paris, and were a gift from Dr. Louis Chedid. All other chemicals were reagent grade. Needles, syringes, glassware, and solutions were sterile and pyrogen-free. Pyrogen-free saline and water were obtained from Abbott, North Chicago, IL.

Animals and Surgery

Male albino New Zealand Pasteurella-free rabbits, weighing 3-4 kg, were used. They were prepared for surgery by injecting a mixture of 35 mg/kg ketamine and 5 mg/kg xylazine in a volume of 0.6 ml/kg subcutaneously. Rabbits were then provided with the following. Stainless steel screws were implanted in skull burr holes as follows (units are in millimeters; bregma is reference;

A = anterior; P = posterior; L = lateral): (1) for cortical EEG, one at A5.0/L2.0 and one at P10.0/L3.0; (2) for nasal bone EEG, one at A20.0/L2.0 and one at A40.0/L3.0; (3) for ground reference, one at P1.0; (4) for support of the thermistor attachment, one at P8.0/L5.0. A glass bead thermistor (Fenwall Elect., No. GA45J1, 50KΩ) was coated with dental acrylic cement for insulation. It was lowered 4.0 mm below the skull surface through a burr hole at P12.0/L3.0. Its estimated depth in cortex was, therefore, 1.0 mm. A cerebral ventricular guide tube was also implanted (P0.0/L2.7) as previously described (56, 120).

In addition, in some cases, one or both of the following implants were made: (a) for vibrissae electromyograms (EMG), two Teflon-coated wires (#316 SS 5T, Medwire Corp., NY) were passed under the skin, one from each side of the snout, to the top of the skull; or (b) for electro-oculogram (EOG) electrodes, two Teflon-coated wires were exposed at one end, which was folded to make a hook and inserted into the connective tissue over each eyeball.

Insulated wires from Amphenol pins (No. 220-PO2) and miniature plugs (Amphenol No. 223-1509) were soldered to the EEG and thermistor and EMG or EOG leads. The leads and guide tube were secured to the skull with dental cement (Coralite Dental). After surgery, bacitracin-neomycin-polymyxin ointment (Pharmaderm, NY) was applied under the skin edge surrounding the implant. Gentamicin sulfate (Tech America, KS) was injected intramuscularly at 5 mg/kg, and the rabbit was returned to its cage to recover for at least one week.

Apparatus and Recording

The recording chambers were temperature- and light-controlled (Forma Scientific, model 20, or Hotpack, model 352600). Unless otherwise specified, temperature was kept at 21.0 ± 2°C and light at 100 Lux. A light:dark cycle of 12 h:12 h was maintained, light from 0600 to 1800 h. Food and water were available ad libitum. At the top of the chamber, a BRS/LVE electrical contact swivel was fixed which allowed the rabbit free movement during recording periods. From the other end of the swivel, a cable led to a Grass polygraph, model 7D.

Before each recording period, animals were connected to the recording cable for a 1-h habituation period. Rabbits were then briefly taken out of the experimental cage and given the test substance. When animals received intracerebroventricular (ICV) injection, test substances were dissolved in 50 μl of artificial CSF [(in mM) 3 KCl, 1.15 $CaCl_2$, and 0.96 $MgCl_2$ in nonpyrogenic saline, 155 mM NaCl (Abbott)].Solutions were then slowly infused over 5 min into a lateral ventricle. If test substances were administered intravenously (IV), they were dissolved in 0.5 ml of saline, then injected into a marginal ear vein. After injection, colonic temperatures were taken. Rabbits were returned to the experimental cages, and a 6-h recording period began. After the recording period, colonic temperatures were taken again.

EEG, brain temperature (T_{br}), and movement were recorded from all animals to help define states of vigilance (see below). Other measurements were also taken; these were vibrissal EMG, EOG, nasal bone EEG (respiratory EEG), and ratios of theta/delta cortical EEG activity. Similar measurements were

previously described in studies of rabbit sleep (6, 91). To determine T_{br}, implanted thermistors were calibrated using colonic temperatures; this method assumes that T_{br} follows colonic temperature within a constant range. On an experimental day when an animal received a pyrogenic substance, colonic temperatures were taken before and after fever developed while simultaneously recording T_{br}. The difference between the two T_{br}s on the polygraph paper was assumed to be equal to the difference in the two colonic temperatures, thus allowing quantification of T_{br} at other times. Rabbit body movement was monitored by means of a pressure transducer as previously described (57) or by using a Grass tremor transducer attached to the recording cable. In some cases, the cortical EEG signal was also fed into a Buxco (Sharon, CT) integrator-averager, model 180, system. The analyzer separated the EEG into several frequency bands; delta (0.5-3.5 Hz), theta (4-7 Hz), alpha (8-12 Hz), and beta (15-20 Hz). The ratios of voltages in the theta and delta bands were continuously computed and displayed on polygraph paper simultaneously with the EEG. In addition, the nasal bone EEG was filtered; its delta activity was rectified using a Buxco model 188, and this rectified delta activity was also recorded on the polygraph paper.

Identification of States of Vigilance

REM sleep was characterized by low-voltage EEG, high theta/delta ratios, a rapid rise in T_{br}, and movement twitches. SWS was characterized by high amplitude EEG, low theta/delta ratios, decreased T_{br}, little or no movement, and a mid-level nasal bone EEG delta activity. Wakefulness (W) was defined as all periods not classified as SWS or REM sleep. W was associated with a low voltage EEG, movement, high EMG activity, high nasal bone EEG delta activity

and mid-level cortical EEG theta/delta ratios (see Figs. 1 and 2 for recording samples).

Quantitative Analysis

The polygraph strip charts were scored with the use of a digitizer board and its associated driver software (Jandel Scientific) on a Zenith Z-148 microcomputer (MS-DOS v3.1 operating system). The charts were placed on the board, and trace distances measured to the nearest 2.5 mm using a stylus-type pointing device. Each distance measured was labeled as either slow-wave sleep, rapid eye movement sleep, wakefulness, or noise. Data were automatically stored on flppy disks: one file for each hour of the experiment. The six raw data files were then used by a compiled custom-written Turbo Pascal program to produce totals for each sleep type for each hour, percentages of each sleep type for each hour, colonic temperature differences, and a data file which is a compilation of the raw data files. This file holds raw data, but in a corrected and more efficient format for archival and subsequent retrieval purposes.

Statistical analyses included multivariate analysis with repeated measures, Student's t-test for paired data, Student's t-test for unpaired data, and chi-square analyses. A significance level of $P < 0.05$ was used. Specific statistical analyses used are indicated where appropriate in Results.

SPECIFIC EXPERIMENTAL PROTOCOLS

Experiment No. 1: Effects of MDP on Normal Rabbit Sleep at Different Ambient Temperatures and Different Times of the Day

A. MDP vs. MDP-DD

A group of five rabbits was subjected to all experimental and control conditions (complete within-subject, repeated measures design) for this study. Each received injections of MDP, which is somnogenic, and a steroisomer MDP-DD, which is not somnogenic (63) as control. Each rabbit was allowed a day of habituation to the recording chamber during which data were not collected. On subsequent days, recordings were obtained from each animal between 0900 and 1500 h. Each animal received four treatments on separate days; the order of treatment was different for each animal. Treatments were: A1) no infusion control; A2) ICV infusion of artificial CSF; A3) ICV infusion of 100-125 pmol MDP; A4) ICV infusion of MDP-DD. The numbers A1-A4 are used in the tables and figures in reference to treatment groups.

B. Effects of elevated ambient temperature (T_a)

The same group of rabbits subjected to the experimental protocol A above were also used to determine the effects of elevated T_a on rabbit sleep. Each animal (n = 5) was exposed to three conditions; thus, a within-subjects, repeated measures design was used. Conditions were B1) no infusion, 27°C; B2) artificial CSF infusion, 27°C; and B3) ICV infusion of 125 pmol MDP, 27°C. Ambient temperature was reset from 21°C to 27°C at the beginning of the 6-h recording session just after ICV infusions of the test solution or at the onset of recordings. The choice of 27°C was guided by: a) the literature concerning rabbit thermoneutral zones (40) and our own observation that this was a threshold for panting and b) the evidence that when febrile, rabbits prefer warmer ambient temperature (100). After each session, the animal was returned to the animal colony, which was kept at 21°C.

C. Effects of MDP during light and dark phases

Two doses of MDP, 5 μg/kg and 25 μg/kg given IV, were used in both the light and dark phases. Control was "no injection." Recording during the light session took place from 1000 h to 1600 h. During the dark session, they were obtained from 1800 h to 2400 h. At least one week of rest separated successive administrations of MDP. Thus, there was a total of six conditions as follows: 1) light, no injection; 2) light, MDP, 5 μg/kg; 3) light, MDP, 25 μg/kg; 4) dark, no injection; 5) dark, MDP, 5 μg/kg; and 6) dark, MDP, 25 μg/kg. For the dark phase testing, eight rabbits were exposed to all treatments. Thus, a within-subjects, repeated measures design was used. For the light phase, at least eight rabbits were in each group. However, some rabbits (four) did not receive both doses of MDP. Thus, in this case, a between-subjects design was used.

Experiment No. 2: Interaction of MDP with Amphetamine and Eserine

Drugs

MDP or its vehicle control (artificial CSF) was infused ICV at a rate of 10 μl/min, just before the beginning of a six-hour recording session. Amphetamine (Sigma) was dissolved in saline; a fresh solution was prepared each week. Amphetamine was injected subcutaneously (SC) in the back, 1.0 mg/kg in a volume of 0.5 ml. Saline (0.5 ml) was injected as a control. Eserine (Sigma) was dissolved in saline 30 min before IV injection. It was administered at a dose of 0.1-0.15 mg/kg into the marginal ear vein. Both eserine and its saline control were administered in a volume of 0.5 ml. Doses of amphetamine and eserine were similar to those previously used (21, 38, 49, 76).

Experimental Protocol

The design of both the amphetamine and eserine experiments was repeated measures: each rabbit was exposed to all experimental and control conditions, except that in testing a high dose of MDP (12,500 pmol) for interaction with amphetamine, we used between-groups comparisons. To control for order effects in the eserine experiment, treatment order was partially counterbalanced. In the amphetamine experiment, order effects were not controlled, but repeated recordings were made using vehicle SC controls for the effect of repeated injections alone.

A. MDP and Amphetamine

The amphetamine protocol was as follows: (a) day 1, saline SC; (b) day 2, MDP (125 pmol ICV) + amphetamine SC (1 mg/kg); (c) day 3, saline SC; (d) day 11, saline SC; (e) day 12, CSF ICV + amphetamine SC (1 mg/kg); (f) day 26, saline SC; (g) day 27, MDP 12,500 pmol ICV + amphetamine SC (1 mg/kg); (h) day 28, saline SC. All ICV infusions were made just before the start of a six-hour recording session. All SC injections were made just after the end of the first hour.

B. MDP and Eserine

The eserine protocol was as follows. All ICV infusions were made just before the start of a six-hour recording session. All IV injections were made just after the end of the second hour. Treatments were (a) no infusion, (b) saline IV, (c) eserine (0.15 mg/kg IV), (d) MDP (125 pmol ICV) + saline IV,

(e) MDP (125 pmol ICV) + eserine (0.15 mg/kg IV). Time between treatments varied between 4 and 14 days. Minimal time between ICV infusions was one week.

Experiment 3: Effects of Various Muramyl Peptide Analogs Derived from Bacteria on Sleep

A. Growth of Bacteria and Preparation of Muramyl Peptides

Neisseria gonorrhoeae was grown as described (98) at 37°C in liquid medium LGCB$^+$ (pH 7.3) containing 0.4 % (wt/vol) pyruvate and 0.012 μCi/ml of D-[1-^{14}C] glucosamine (ICN Pharmaceuticals, Inc., Irvine, Calif.). Purified intact (insoluble) peptidoglycan was purified from exponential phase bacteria by a trichloroacetic acid-sodium dodecyl sulfate extraction procedure (41) as modified (109) to include (i) extraction with sodium dodecyl sulfate at pH 5.1 and (ii) treatment of the sodium dodecyl sulfate-insoluble residue with proteinase K. The final, washed insoluble material (intact peptidoglycan) contained less than 0.9% (wt/wt) non-peptidoglycan amino acids.

Purified intact peptidoglycan was used as starting material for two structurally-related families of monomeric MPs. Each of these sets, which are referred to as "Chalaropsis monomers" and "anhydro monomers", respectively, was initially isolated as mixtures of monomeric MPs that were ultimately used as the sources of final MPs used in these studies. Chalaropsis monomers were isolated by gel filtration on connected columns of Sephadex G-50 and G-25 after complete digestion of intact peptidoglycan with Chalaropsis B muramidase

as we have described previously (98). Pooled monomeric fractions were desalted by gel filtration on Sephadex G-15 eluted with pyrogen-free water. Chalaropsis monomers served as the main source of the set of MPs with hydrated, reducing NAM ends.

Anhydro monomers were prepared from intact peptidoglycan with use of a partially purified enzyme preparation obtained from Escherichia coli ATCC 9637. This preparation contained both DD-endopeptidase and peptidoglycan: peptidoglycan-6-muramyl transferase (transglycosylase) activities. Several procedures for purification of these peptidoglycan hydrolases have been reported (43, 71, 84). For our purposes, the optimal procedure was an unpublished method (personal communication, U. Schwarz, Tubingen, Federal Republic of Germany) in which the key step involved chromatography on CM-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) of Triton X-100 extracts of sonicated E. coli. Details of the protocol for the complete digestion of intact peptidoglycan with the E. coli transglycosylase-endopeptidase have been published previously (83). Ultimately, anhydro monomers were isolated from the peptidoglycan digest by gel filtration and were desalted as for Chalaropsis monomers. Using this procedure, the percent yield of anhydro monomers from intact peptidoglycan as starting material was exceptionally high (about 60%). The efficiency of this reaction was attributed to the virtually complete conversion of insoluble peptidoglycan to anhydro monomers by the novel use of an enzyme preparation which contained both glycan-splitting (transglycosylase) and peptide-splitting (endopeptidase) activities. Anhydro monomers served as the main source of MPs with non-reducing, 1,6-anhydro-NAM ends.

At completion, both Chalaropsis monomers and anhydro monomers contained no detectable non-peptidoglycan amino acid contaminants at a sensitivity that should have easily detected less than 0.1% (wt/wt), and no more than 0.012 ng of gonococcal endotoxin per μg of purified monomeric peptidoglycan, as determined by Limulus amoebocyte lysate assay performed according to the manufacturer's instructions (Associates of Cape Cod, Woods Hole, Mass.).

B. High Performance Liquid Chromatography and Fast Atom Bombardment-Mass Spectrometry

Chalaropsis monomers and anhydro monomers, prepared as described above, were further purified using a Waters HPLC model 6000A binary pumping system with solvent programmer and a Waters μBondapak C-18 reversed-phase column. A Beckman model 160 absorbance detector operated at 214 nm and a Hewlett Packard 3390A integrator were used for detection. The solvent program used for separation was linear form 0 to 10% CH_3CN (containing 0.035% CF_3COOH) in water (containing 0.05% CF_3COOH) over a period of 25 min at a flow rate of 1.5 ml/min. Elution peaks were collected and analyzed as described below. To help verify structures, in some cases, aliquotes of subtances were derivatized or modified before mass spectral analyses. These steps involved methylation of free carboxyl groups and acelyation of hydroxyl and amino groups as described in detail elsewhere (78, 79).

A double focusing (Varian MAT 731) mass spectrometer of the Mattauch-Herzog geometry with a mass range of 1-2000 daltons at 8 kV accelerating potential was employed in the work (78). Both an Ion tech B12N 100 μA neutral

beam source and a JEOL ion/neutral beam source were used with Xe as the bombarding species. The samples were dissolved in aqueous acetic acid (ranging from 10 to 50%) to which an equal volume of glycerol was added. Approximately 0.5 μl of this mixture was applied to a stainless steel probe tip attached to a high vacuum push rod and inserted into the ion source housing of the mass spectrometer. The push rod allows positioning of the sample probe tip within the ion source such that it intersects the path of the Xe particle beam. The ions produced by the Xe beam's interaction with the sample on the probe tip are accelerated, focused, mass selected, and detected. Exact mass measurements were made in the peak match mode (at a resolution of 1/10,000) employing well-characterized reference peptides whose exact mass was within 10% of mass of that of the unknown compound. The reference peptide was added directly to the sample on the probe tip so that the concentration ratio of sample to reference peptide was approximately 3/1. Additional reference peptide could be added to the probe tip in order to produce ion signals of equal intensity. [Sar^1,Ala^8]angiotensin II protonated molecular ion (MH+) 926.5212 was used as the reference peptide in this study.

Sleep Assays

All the assays in the present investigation were carried out on male rabbits as described above. Samples for assay were dissolved in sterile pyrogen-free artificial CSF (155 mM NaCl, 0.96 mM $MgCl_2$, 1.15 mM $CaCl_2$, 3 mm KCl) and injected ICV. Rectal temperatures were taken immediately after the infusion and 3 and 6 hours later. Individual control values for duration of SWS, REM sleep, brain temperature, and rectal temperatures were determined for each rabbit during 1 or more 6-hour recordings; thus a within-subject experimental

design was used. Each substance was tested in a minimum of four animals; in most cases, two doses of each MP were tested.

Experiment No. 4: Effects of MDP on Sleep, Body Temperature, and Plasma Copper

A. Injections

When animals received ICV injections, test substances were dissolved in 50 μl of artificial CSF [(in mM) 3 KCl, 1.15 $CaCl_2$, and 0.96 $MgCl_2$ in pyrogen-free saline (155 mM NaCl) (PFS), (Abbott)]; these solutions were slowly infused over 5 min. When test substances were administered IV, they were dissolved in 0.5 ml of PFS and injected into a marginal ear vein. Immediately after injection, colonic temperatures were measured; then the rabbits were returned to the experimental cages, and a 6-h recording period was begun. After the recording period, colonic temperatures were measured again.

B. Experimental protocols

The following treatments were given in this study:

1. Naive (n = 12). These were not prepared for sleep recording or ICV infusion and were not injected with any drug or PFS.

2. Operated (prepared for sleep recording and ICV infusion) but never injected with any drug (n = 6, some of which later received MDP). This group, in conjunction with the next, served as controls for the possible effects on

the acute-phase Cu response of any inflammatory reaction consequent to the implantation of electrodes or to any aspect of surgery.

3. PFS ICV (n = 8). These rabbits were also prepared for sleep recording and were monitored in the same fashion as MDP-treated rabbits.

4. MDP, 150 pmol ICV (n = 11). This dose was chosen based on previous experiments in which it was shown to induce fever and enhance SWS (63). Four of these rabbits were part of group 3 above; in all cases, PFS was injected before MDP; at least two weeks were allowed between PFS and MDP injections.

5. MDP, 15 nmol ICV (n = 7 [different rabbits from group 4]). This dose was used to obtain a sufficient range for a dose-dependent pattern.

6. MDP, 15 nmol IV (n = 6 non-implanted rabbits). Since treatment 5 above is such a high dose that fever often persists for more than 25 hours after MDP ICV infusion, we used the same amount of MDP given IV to control for the possibility that some MDP, after ICV injection, passed into the circulation and acted peripherally.

Blood samples were taken between 27-28 hours (1300-1400 h) after MDP treatment (or control infusions) or at 1300-1400 h on any day in the case of the "naive" or "operated only" groups. Acute-phase glycoproteins are maximally deviated from their baseline levels 24-28 h following lipopolysaccharide or interleukin-1 administration (12, 13). Blood was withdrawn from a marginal ear vein into 3-ml glass syringes precoated with saturated sodium citrate as the anti-coagulant to a volume of 2.0-2.5 ml. The sample was collected in

Falcon tubes (#15) coated with sodium citrate (100 μl) and then subjected to centrifugation (1,500 x g, 15 min). The plasma was stored frozen at -20°C.

C. Quantitative Analyses

To determine plasma Cu levels, the frozen plasma samples were thawed at room temperature, diluted 1:20 with demineralized water, and analyzed for Cu by high temperature, flameless atomization, atomic absorption spectrophotometry using the Standard Addition method as previously described (13). Results are expressed as μg Cu/dl plasma. Statistical significance of differences between treatments was tested by Student's t-tests for independent groups.

Experiment No. 5: Effects of recombinant tumor necrosis factor (rTNF) and recombinant interleukin-1 (rIL1) on Rabbit Sleep

Human rTNF was expressed in Escherichia coli, purified to homogeneity (90) and was provided by Genetech, Inc., (So. San Francisco, CA) in phosphate-buffered saline containing 500 μg protein/ml. Solutions of rTNF were assayed for the presence of endotoxin using the Limulus amoebocyte lysate assay (Mallinckrodt, St. Louis, MO). Less than 200 pg of endotoxin per mg of rTNF were found (31). The rIL1 used in these studies was the pI 7 (or beta) form (3) and was provided by Cistron Technology, Inc. (Pine Brook, NJ) in phosphate-buffered saline containing 500 μg protein/ml. Recombinant IL1 was expressed in E. coli and consisted of amino acids 112-269 of the precursor sequence (32). The rIL1 was also tested for the presence of endotoxin, and between 40-80 pg endotoxin per mg protein were found.

Before each recording period, the rabbits were connected to the recording cable for a 1-h habituation period, but data were not collectd during this time. Rabbits were then briefly taken out of the experimental cages and given the test substance. When animals received ICV injections, appropriate amounts of test substances (0.1-10 μl) were diluted to 50 μl with artificial CSF; these solutions were slowly infused over 5 min. For IV injection, aliquots of rTNF or rIL1 (2-60 μl) were diluted to 0.5 ml in PFS and then rapidly (30 sec) injected into a marginal ear vein. Immediately after injection, colonic temperatures (T_{co}) were measured using a calibrated thermistor probe (Yellow Springs Instruments) inserted 10 cm into the colon; then the rabbits were returned to the experimental cages for a 6 h recording period. After the recording period, T_{co} was measured again. Injection (IV or ICV) took place between 0800 and 1000 h. A total of 36 ICV injections of experimental samples was given to a group of 26 rabbits. A total of 21 IV injections was given to 14 rabbits; all of these rabbits were part of the ICV-injected group. After an injection, at least 1 wk was allowed before a rabbit received another injection. Two rabbits received four injections of the same substance; in these cases, no signs of tolerance developed. For example, in response to rTNF (5 μg, ICV), a rabbit spent 65% of the time in SWS; one month later, in response to rTNF (10 μg/kg, IV), the same animal spent 79% of the time in SWS, compared to a control value of 47% SWS. On separate days, control recordings were obtained from the same animals without injection; therefore, a within-subject experimental design was used. Previously, we showed that ICV or IV injections of control solutions of artificial CSF do not disturb subsequent sleep in rabbits (61). For ICV infusion, doses are expressed in grams because adult rabbit brains weigh about 8 g, regardless of body weight.

RESULTS

Experiment No. 1: Effects of MDP on Normal Rabbit Sleep at Different Ambient Temperatures and at Different Times of the Day

Experiment A: MDP vs. MDP-DD

ICV administration of artificial CSF or MDP-DD failed to significantly affect any of the sleep or temperature parameters measured in this study (Tables 1 and 2). In contrast, the duration of SWS and T_{br} was significantly elevated by MDP (Fig. 3, Table 1). During the first hour postinfusion, duration of SWS was significantly reduced by MDP; from the second hour through the sixth hour postinjection, duration of SWS was significantly elevated (Fig. 3). Enhanced SWS resulted from a significant increase in the number of SWS episodes of longer duration (Table 2I), as indicated by chi-square analyses comparing column A3 to column A4. REM sleep was significantly reduced after MDP administration (Table 1, Fig. 3). This reduction of REM sleep resulted from a significant reduction in the number of REM episodes (Table 2II, A3 vs. A4).

Although we did not make quantitative behavioral observations, behavior of animals after MDP treatment appeared normal. Animals continued to eat, drink, and groom. Sleep postures were normal, and animals responded to external stimuli. Further, we failed to observe any autonomic side effects after MDP injections at the doses used. Although animals were febrile after MDP treatment (Table 1, Fig. 3), T_{br} changes that are normally associated with changes in state of vigilance, e.g., increased T_{br} after entry into REM sleep, persisted in MDP-treated animals (data not shown).

B. MDP Effects during Acute Elevation of T_a

The effect of acute elevation of T_a (21°C to 27°C) was to increase duration of SWS (Table 1, Fig. 4). Unlike the effects of MDP given at 21°C, increasing T_a to 27° increased duration of SWS during the first hour of recording (Fig. 4). Increased SWS resulted from an increase in the number of SWS episodes of longer duration (Table 2I). REM sleep duration (Table 1) and the frequency of occurrence of REM sleep episodes of various durations (Table 2II) were not significantly affected by increased T_a from 21°C to 27°C. Brain temperatures were consistently lower in the higher ambient temperature condition (Fig. 4), although this difference was not significant.

At 27°C MDP significantly increased SWS above control values obtained at the same T_a (Fig. 5, Table 1). This summative effect was also expressed in the distribution of SWS episode lengths (Table 2I). Increasing T_a alone increased SWS by significantly shifting the distribution toward episodes of longer duration (Table 2I; columns B1 vs. A1); MDP further magnified this effect (Table 2I, columns B2 and B3). At the higher T_a, the MDP-induced reduction of REM sleep persisted, and brain temperatures were elevated significantly (Table 1 and Fig. 5).

C. Effects of MDP during the Light and Dark Phases

Rabbits slept less during the dark than during the light phase under control conditions, although this reached significance only for REM sleep (Table 3). Brain temperatures were consistently elevated during the dark compared to

the light phase, thus confirming an earlier report (73), but these differences were not significant (Table 3).

During the light phase, IV administration of MDP at a dose of 25 μg/kg increased duration of SWS and fever and suppressed duration of REM sleep (Table 3, Fig. 6). These effects and the time course of these effects were similar to those observed after ICV administration of MDP (compare Figs. 3 and 6). Further, the increase in SWS and reduction of REM sleep resulted from an increase in number of SWS episodes of long duration and a reduced number of REM sleep episodes (data not shown) as described above for data obtained after ICV injection of MDP. During light hours, injection of the lower dose of MDP, 5 μg/kg, did not significantly increase duration of SWS for the 6-h postinjection period (Table 3).

During the dark phase, the effects of 25 μg/kg MDP on SWS were similar to those observed after injection of MDP during light hours (Fig. 6), although duration of SWS for the 6-hour postinjection period was not significantly enhanced. However, if we restrict our analysis to hours 1-5 postinjection, the 25 μg/kg dose of MDP significantly increased duration of SWS during the dark hours (67% experimental vs 50% control, $P < 0.005$). There were no significant effects of this dose of MDP on REM sleep when compared to no injection control values obtained during dark hours (Table 3).

The effects of MDP on brain temperatures were substantially different in the dark than in the light. During light hours, 5 μg/kg IV MDP induced fever; the same dose at night was without effect on brain temperature (Table 3). The higher dose of MDP (25 μg/kg) induced long-lasting fever during light hours

(Fig. 6). During dark hours, this dose of MDP induced a transient increase in brain temperatures which lasted only about two hours (Fig. 6).

Experiment No. 2: Interaction of MDP with Amphetamine and Eserine

A. Interaction of MDP with Amphetamine

Repeated injections of control solutions and the particular design which we chose did not affect any of the sleep parameters we measured or T_{br} (Fig. 7A, Table 4). Administration of artificial CSF + amphetamine reduced duration of SWS (Fig. 7A) for two hours following injection. During this two-hour period, the decrease of SWS was due to a reduction of the average SWS episode length and the number of SWS episodes, although the latter reduction was not significantly changed (Table 4). REM was completely inhibited for two hours after amphetamine administration, then recovered to control values (Fig. 7A). Amphetamine did not induce behavioral hyperactivity at the doses used. Brain temperatures were slightly, but not significantly, elevated after amphetamine injection (Table 4).

If MDP was administered one hour before amphetamine (125 pmol), duration of SWS was restored to control levels for the two-hour period after amphetamine injection (Fig. 7A). During the next three hours (hours 4, 5, 6, Fig. 7A), SWS was enhanced above values obtained after control and amphetamine treatments. The MDP-induced restoration of SWS resulted from an increase in the number of SWS episodes while the mean episode length remained low compared to controls (Table 4). MDP at a dose of 12,500 pmol had a similar but more potent effect on amphetamine-induced hyposomnia. During the second hour after

infusion, SWS was not only restored, but was significantly elevated above saline control levels (Fig. 7B). Mean SWS episode length was restored after the higher MDP dose, and the number of SWS episodes was enhanced (Table 4). The day after the high MDP dose, duration of SWS (Fig. 7B) and length of SWS episodes (Table 4) were close to control values.

The effects of MDP treatment in combination with amphetamine on REM sleep are shown in Fig. 7. As mentioned above, amphetamine by itself induced a transient (2 hour) REM sleep inhibition; MDP (125 pmol) partially restored REM sleep during this period, but not to control levels (Fig. 7A). In fact, after the low dose of MDP, REM sleep inhibition was prolonged, extending into hour 5. The high dose of MDP induced a more prolonged inhibition of REM sleep duration to the extent that the day after MDP injection, duration of REM sleep remained below control values (Fig. 7B). Both doses of MDP also induced increases in T_{br} above the amphetamine-induced elevated T_{br} (Table 4). As in the case of REM sleep duration, the effect of the higher dose of MDP on T_{br} persisted into the day after injection (Table 4). In a separate group of animals injected only with 12,500 pmol MDP, we found that this long-lasting temperature effect is from MDP and not from an interaction with amphetamine (data not shown).

The interaction of MDP and amphetamine caused a transient dissociation of T_{br} and nasal bone EEG fluctuations from states of vigilance. Brain temperature did not fluctuate with transitions between waking and SWS episodes, although later during the six-hour recording period the relationship between T_{br} and sleep states recovered. The higher dose of MDP in combination with amphetamine induced a longer-lasting dissociation of T_{br} and sleep states

and, in addition, nasal bone EEG delta waves persisted after transition from W to SWS (judged by immobility and cortical high voltage waves), whereas in the normal rabbit this activity diminishes during such transitions (Fig. 2).

B. MDP Interaction with Eserine

Eserine alone reduced SWS and REM sleep (Fig. 8B). Immediately after the intravenous infusion of eserine, there followed a period of 50-60 min of continuous wakefulness as judged by the presence of cortical low voltage fast EEG and motor (behavioral) activity. During the first 30 min, the motor activity consisted of restlessness while standing in one place. There appeared to be several small muscular contractions and some tremor. Then this motor activity subsided, and the rabbits engaged in more normal motor activity. During the first hour after eserine treatment, REM sleep was also inhibited (Fig. 8B).

MDP failed to reverse eserine-induced inhibition of SWS and REM sleep. When eserine was administered at the end of the second hour after MDP ICV infusion, it induced a period of cortical low voltage fast EEG as long as that observed after eserine was administered alone (Fig. 8B). Fever induced by MDP was not affected by eserine (Table 4), and T_{br} fluctuated with changes in sleep states as they did after the control treatment (data not shown).

Experiment No. 3: Effects of Various Muramyl Peptide Analogs Derived from Bacteria on Sleep

The O-acetylation of muramic acid in NAG-(6,OAc)NAM-Ala-γGlu-A_{2pm}-Ala enhanced SWS as compared to the respective non-O-acetylated compound (Table

5). The amidation of the terminal alanine of NAG-(1,6-anhydro)NAM-Ala-γGlu-A_{2pm}-Ala(NH_2) prevented the otherwise very strong SWS-enhancing activity of this molecule (64). It was previously demonstrated that prevention of SWS enhancement also could be achieved by amidation of the E-carboxyl group of A_2pm in this molecule (67).

The MP-dimers showed no SWS-promoting activity. However, the dimer containing a (1,6-anhydro)NAM was active at the very high dose of 100 pmol. If this was due to an intrinsic activity of this dimer, or to a contamination of this dimer with the respective, very active, monomer (64), was not investigated.

Experiment No. 4: Effects of MDP on Sleep, Body Temperature, and Plasma Copper

Plasma Cu levels of naive unoperated rabbits were similar to those obtained from operated animals (Table 6). In contrast, PFS injected ICV caused a significant reduction of Cu plasma levels compared to operated control levels. However, PFS did not affect either duration of SWS or body temperature, although it induced a reduction of REM sleep. In contrast, MDP given ICV had profound effects on plasma Cu levels, sleep, and body temperature (Table 6). The lower dose of MDP, 150 pmol ICV, induced significant increases in plasma Cu levels, duration of SWS, and body temperature. This dose of MDP also greatly reduced REM sleep. These effects were magnified to a greater extent after ICV injection of the higher (15 nmol) MDP dose. To determine if the effects of MDP on plasma Cu levels were mediated via a central mechanism or were the result of some MDP entering the systemic circulation from the brain,

15 nmol MDP was injected IV, and plasma Cu levels and body temperature were determined. This dose of MDP, given IV, failed to affect either plasma Cu levels or body temperature.

Experiment No. 5: Effects of rTNF and rIL1 on Rabbit Sleep

A. Effects of rTNF on sleep and EEG voltages

Recombinant TNF enhanced SWS after IV or ICV administration (Table 7, Fig. 9). After the ICV injection of 5 μg rTNF, enhanced SWS was observed throughout the 6-h recording period. After ICV injections of 0.5 μg rTNF, SWS was not significantly enhanced for the 6-h postinjection period (Table 7). However, if we confine our analyses to the first h postinfusion, this dose of rTNF significantly enhanced SWS (Fig. 9). After IV administration of rTNF, SWS was not significantly increased during the first 2-h postinjection (Fig. 9). However, after IV administration of 10 μg/kg rTNF (about 30 μg/rabbit), significant increases in SWS were observed during the last 4 h of recording (Fig. 9). In contrast, rTNF inhibited REM sleep after either IV or ICV injections of rTNF in amounts sufficient to significantly enhance SWS for the 6-h recording period (Table 7, Fig. 9). It is noted that after the ICV dose of 0.5 μg rTNF, REM sleep was not inhibited during the first h after injection when SWS was enhanced (Fig. 9). The behavior of animals that received rTNF appeared normal in that animals continued to spontaneously awaken; during W episodes, normal behavior such as eating and grooming was observed. In addition, after higher doses of rTNF, when duration of REM sleep was less, bouts of REM sleep continued to alternate with episodes of W and SWS as was observed in control animals. Sleep postures were also normal after rTNF treatment.

During recovery sleep after sleep deprivation, amplitudes of EEG slow waves are enhanced (88). A similar effect on amplitudes of EEG slow waves was observed during bouts of SWS after administraton of rTNF. These enhanced EEG amplitudes were observed after both ICV doses of rTNF and after the 10 μg/kg IV dose (Table 7).

Recombinant TNF also increased the average delta wave voltages observed during the 6 h recording periods (Table 7). Results expressed as average voltage over long time periods are not subject to biases that individuals may have during scoring of polygraph records. They do not necessitate the identification of vigilance states, and they are electronically computed. Increases in average voltage result from an increase in the amplitudes of EEG delta waves; high amplitude delta waves are associated with bouts of SWS. Thus, average delta voltages for the 6-h postinjection period were significantly enhanced after ICV administration of 5 μg or IV injections of 10 μg/kg rTNF. Voltages in other frequency bands (alpha, beta, theta) were also determined after rTNF treatment; none of these voltages were significantly affected by rTNF (data not shown). In addition, after 0.5 μg rTNF (ICV), enhanced average delta voltages were observed during the first h postinjection (Fig. 9). This result, therefore, reinforces those obtained by visual scoring of the records in that ICV rTNF enhanced (0.5 μg) SWS during the first h after injection.

B. Effects of rIL1 on rabbit sleep EEG voltages

Human rIL1 enhanced SWS after either IV or ICV administration in a dose-dependent manner (Table 8, Fig. 10). After ICV administration of rIL1, enhanced SWS was observed during the first h after injection. However, after IV

administration of somnogenic doses of rIL1, there was a delay of about 1 h before the onset of excess SWS was observed (Fig. 10). In addition, those doses of rIL1 that significantly enhanced SWS also significantly inhibited REM sleep, thus confirming the previous findings that native IL1 can inhibit REM sleep (113). Other aspects of sleep and behavior were normal, as described above for rTNF-induced responses.

Two different lots of rIL1 were used (Table 8). In both cases, responses were dose-dependent, although lot No. 2 was more potent than lot No. 1; e.g., after ICV injection of 50 ng of lot No. 2, there was a significant increase in SWS, whereas this dose of lot No. 1 was inactive with regards to SWS. We also tested a very high dose of lot No. 2 (5,000 ng, ICV). Large sleep and fever responses were observed after this dose. However, after the 6-h recording period, behavior of these animals was abnormal in that they were not very responsive to handling and they were panting. Also, one of the animals died three days after injection of this dose.

The effects rIL1 (lot No. 2) had on EEG voltage in the delta and other frequency bands were also determined. Under control conditions, there was little variation in voltage in any of the frequency bands over the 6-h recording period (Fig. 11). After the rIL1-treatment, there was a relatively large increase in average voltage in the delta frequency band. The increase average delta voltage resulted from both an increase in duration of SWS and increased amplitude of EEG slow waves (data not shown) as described above for rTNF. A significant increase in theta voltages was also observed, but this increase was relatively small compared to that observed in the delta band. Recombinant IL1 failed to affect average voltages in either alpha or beta frequency bands.

Values from the beta band are not shown because their magnitude and variations were indistinguishable from those in the alpha frequency band.

C. Effect of rTNF and rIL1 on brain temperature

Both rTNF and rIL1 induced febrile responses in rabbits after ICV or IV administration. However, rTNF induced biphasic febrile responses whether it was given IV or ICV. In contrast, rIL1-induced fever responses were monophasic (Fig. 12), thus confirming previous findings (31). Further, after IV rIL1, febrile responses were much shorter than those observed after IV rTNF injections.

Of particular importance is that sleep responses induced by either rIL1 or rTNF were not tightly coupled to febrile responses. For example, after IV rIL1, T_{br} was close to control values 3-4 h after injection; during this period, maximum SWS responses were observed (compare Figs. 10 and 12). In addition, the lower doses of rTNF, ICV or IV, induced fevers without inducing prolonged increases in excess SWS. The separation of fever and SWS responses is particularly evident when comparing the time course of ICV (0.5 μg) rTNF-induced SWS and T_{br} changes. During the first h postinjection, excess SWS and increased T_{br} were observed. However, during hs 3-5 postinjection, SWS values were close to control values while animals were febrile (Figs. 9 and 12). Similarly, the time course of febrile responses (Fig. 12) did not correlate with either rTNF- (Fig. 9) or rIL1- (Fig. 10) induced REM sleep responses or with the effects of rTNF on delta wave voltages. For example, after ICV injection of rTNF (0.5 μg), average voltages during h 2-6 postinjection were near control values (Fig. 9) although the animals were febrile (Fig. 12). REM

sleep 3-6 h after ICV rIL1 (500 ng) was below control values although animals were not febrile during this period.

Another facet of temperature regulation was unaffected by either rTNF or rIL1. During the transitions between states of vigilance, there are characteristic changes in T_{br}. For example, after entry into REM sleep, there is a relatively rapid rise in T_{br}, whereas during the transition between W and SWS, there is a regulated decrease in T_{br} (42). Previously, we showed that these state-coupled temperature changes persisted in IL1-treated rabbits (120). These T_{br} changes also persisted in rIL1-treated and rTNF-treated rabbits even during periods of hyperthermia (data not shown).

DISCUSSION

Experiment No. 1: Effects of MDP on Normal Rabbit Sleep at Different Ambient Temperatures and Different Times of the Day

The states of sleep usually are defined by a group of physiological processes which vary together (48). It is assumed that a "state" gives rise to a recognizable set of phenomena in these various physiological processes. Thus, even though rabbits do not display a sustained inhibition of neck muscle tone, a major sign of REM sleep in many species, they display other activities unique in their simultaneous appearance which parallel REM sleep of other species (6, 91). We were able, therefore, to develop a definition of REM sleep that was dependent upon cortical EEG, EEG theta/delta ratios, movement, and T_{br} which allowed quantitation of the effects of MDP on REM sleep in rabbits.

Results presented here confirm the earlier findings that MDP is somnogenic and pyrogenic (1, 46, 51, 57, 63, 64, 103, 121). The new findings reported here are: (a) MDP reduces REM sleep in rabbits; (b) elevation of T_a sufficient to promote SWS does not restore REM sleep reduced by MDP; (c) the enhancement of SWS by warmer T_a summates with the MDP enhancement of SWS; and (d) MDP-induced fever is attenuated during the dark phase. We also think it is important to emphasize that in the rabbit, ICV injections of either the vehicle control, artificial CSF, or the inactive stereoisomer MDP-DD did not alter any of the sleep or temperature parameters we measured. In contrast, ICV injections of vehicle controls into other species, e.g., cat (39) and rat (113), induce an inhibition of REM sleep. Thus, in the present study, results concerning REM sleep are not complicated by effects of the infusion procedure.

Results presented here also confirm the previous finding that if D-ala is substituted for L-ala, thus forming MDP-DD, the somnogenic and pyrogenic activities of MDP are lost (63, 67). We show here that the inhibition of REM sleep is also dependent upon the steric configurations of MDP. Previously, we showed that other variations in MP structures can alter their ability to inhibit REM sleep (57). Some MPs, e.g., N-acetylglucosaminyl-1,6 anhydro-N-acetylmuramyl-alanyl-glutamyl-diaminopimelyl-alanine (NAG-1,6 anhydro NAM-ala-glu-dap-ala) (57), depending on dose, enhance SWS but inhibit REM sleep; others can enhance both SWS and REM sleep, e.g., 1,6 anhydro NAM-ala-glu-dap-ala (57); whereas yet another, i.e., NAG-NAM-ala-glu-dap-gly-ala (57), failed to affect SWS but inhibited REM sleep. Thus, it is likely that MP-induced SWS responses are, in part, independent from REM sleep responses.

Regardless of structural requirements, there may be a biological significance of REM sleep reduction by MPs. In several species (16, 37, 52), during

recovery from sleep deprivation, SWS rebounds first at the expense of REM sleep. REM sleep rebound, if it occurs, comes later. Thus, there may be an active process which inhibits REM sleep to establish a priority for SWS following sleep deprivation; we observed a similar priority after MDP treatment. Further, when SWS is promoted in humans by exercise (44) or by passive heat loading (45), REM sleep is unaffected or even inhibited.

To our knowledge, our results are the first demonstration of the effects of elevated T_a on sleep in rabbits, although in other species it is well known that moderate increases in T_a lead to enhanced sleep (85, 99). In our experiments, T_a of 27°C was sufficient to induce panting in rabbits when they were awake and active. During SWS, panting subsided (not quantified). The reduced brain temperatures observed at 27°C T_a are probably related to this heat loss mechanism (21). Acute exposure of other species to high T_a also results in activation of mechanisms of brain cooling (6). Thus, it is possible that the enhanced SWS observed at 27°C is linked to the activation of heat loss responses.

In contrast, the effects of MDP on SWS seem to be separate from its thermoregulatory effects. MDP increased SWS duration beyond the effect of T_a alone (Fig. 6, Table 1). The enhancement of SWS by MDP occurred despite the fact that MDP is pyrogenic and thus should suppress heat loss responses (110). In neonatal rabbits, MDP enhances quiet sleep, the precursor to SWS, without affecting body temperature (27, 28). Coadministration of an antipyretic with MDP attenuates expected febrile responses, but does not affect SWS responses (63). In addition, some MP analogs are pyrogenic, but do not affect sleep, e.g., NAM-L-ala-D-glu (57, 67), thus suggesting that MP-induced SWS responses

are not secondary to fever responses. Further, brain temperature changes that are tightly coupled to sleep states are undisturbed by either MP treatment (57) or elevated T_a.

In a warmer T_a, MDP reduced REM sleep in rabbits. Thus, the MDP-induced REM sleep inhibition was probably not due to displacement of the thermoneutral zone up toward a febrile level. Indeed, previously, we showed that at some doses certain MPs can induce fever without affecting REM sleep, e.g., NAG-NAM-ala-glu-dap-ala (57). Our results are also complementary to those of Masek (50, 51), who blocked fever using antipyretics in rats without restoring REM sleep reduced by MPs. Thus, current evidence suggests that the effects of MPs on SWS, REM sleep, and body temperature are specific and, in part, independent from each other, although the effects of elevated T_a on SWS may be linked to heat loss responses.

During the times we recorded, rabbits slept less at night than during the day; this reached significance only for REM sleep. SWS responses to MDP were similar during both phases. However, MDP-induced REM inhibition was only observed during light hours. In contrast, in rats (80), a sub-pyrogenic dose of MDP induced an increase in both REM sleep and SWS during the dark phase when rats normally sleep little; the same dose was without effect during daytime when rats normally sleep 70-80% of the time.

The selective lack of effect on brain temperature of 5 μg/kg of MDP during the dark phase is similar to results obtained by Satinoff (101). She found that prostaglandin E_2 administered ICV to rats elevated body temperatures to a much greater extent in the light phase than in the dark phase.

However, in our study, 5 μg/kg of MDP failed to elevate temperature to febrile levels during the dark, whereas it was effective during the light. The mechanisms that underlie these differential responses to pyrogen in the light and dark phases remain unknown, although it is likely that they involve intermediate messengers, such as interleukin-1, whose concentration varies with light: dark cycles (117).

Experiment No. 2: Interaction of MDP with Amphetamine and Eserine

The purpose of this study was to set up conditions under which rabbits sleep less, to allow demonstration of sleep-inducing properties of MDP in this species. Amphetamine alone enhanced W at the expense of SWS by reducing duration of SWS episodes and by completely abolishing REM sleep. This was not confounded with behavioral hyperactivity or "stereotypy," which is induced by higher doses of amphetamine (18, 49). MDP restored SWS in amphetamine-treated animals by increasing the number of SWS episodes. It is likely, therefore, that a component of MDP action in rabbits is induction of SWS episodes. Low doses of MDP failed to reverse completely the shortening of SWS episodes induced by amphetamine. This may testify to the ability of amphetamine to disrupt the continuity of sleep or reflect the possibility that the primary action of enhanced MDP is to induce SWS while doing little for sleep maintenance per se. However, it is noted that "maintenance" and "induction" are inferred descriptive processes and not necessarily exclusive. REM sleep reduced by amphetamine was not restored by MDP; this is in agreement with other reports on the tendency of MDP to suppress REM sleep (28, 63, 121).

The exclusive association of behavioral immobility with EEG slow waves and of movement with low voltage fast waves was not changed even by the higher

doses of MDP in combination with amphetamine. Thus, the states of SWS and W remained intact during the interaction of MDP and amphetamine. However, certain characteristic parameters of sleep states were disrupted by this treatment; i.e., T_{br} changes linked to state of vigilance and nasal bone EEG were transiently dissociated from the sleep/wake transition. This may indicate an interaction of amphetamine and MDP on certain autonomic functions, e.g., ear vasomotor responses (6), panting (21), and/or direct action of MDP on smooth muscle (86).

The failure of MDP to diminish the cortical low voltage fast EEG induced by eserine suggests that MDP does not act directly on cortical EEG mechanisms. Rather, as is evident from its ability to restore sleep reduced by amphetamine, it probably acts on the full transition between states of sleep and waking. The dose of MDP we used is close to that needed to induce maximal SWS responses in rabbits (67), and yet MDP failed to reduce eserine-induced EEG low voltage fast activity. These results corroborate a recent study (28) in which MDP failed to affect the EEG of newborn rabbits while it did increase their behavioral quiet (non-REM) sleep. Further, our results separate the effects induced by MDP from those induced by atropine sulfate, which is known for its ability to induce EEG slow waves (18, 122) since atropine diminishes the EEG effects of eserine and completely blocks amphetamine-induced EEG low voltage fast activity (in an immobile animal) [122]. Thus, MDP is unlikely to act as a muscarinic receptor blocker in the brain to achieve its effects on sleep. However, other types of cholinergic involvement cannot be excluded, e.g., non-releasable stores of acetylcholine accumulate in cortex during sleep deprivation (114).

These differential effects of eserine and amphetamine do not lend themselves directly to hypotheses concerning the site(s) of action of MDP in brain. However, studies by Masek et al. (80) and others (75) concluded, based on lesion studies, that MPs act at a midbrain level. Most evidence of the anatomical level of action of eserine and amphetamine comes from brain transections and/or discrete lesions. Thus, transverse transections separated the forebrain, at various levels, from the lower brain. Transection at midbrain-diencephalic level abolishes the effect of amphetamine but not of eserine on the EEG (18, 38). Transections higher than the diencephalic level abolish both amphetamine and eserine effects on the EEG (18, 38, 94). It is tempting, therefore, to speculate that MDP acts at least at the lower midbrain level, one of its actions being suppression of the activating reticular formation.

Experiment No. 3: Effects of Various Muramyl Peptide Analogs Derived from Bacteria on Sleep

Current results expand our knowledge concerning muramyl peptide structure-somnogenic activity. We showed that O-acetylation of the 6-carbon of muramic acid enhanced somnogenic activity of a MP. Considering the similar effect of (1,6-anhydro)NAM on somnogenic activity of MPs, we emphasize that small substitutions on the C_6 atom of muramic acid greatly modify somnogenic activity. Substitution at this position results in a restriction of stable conformational forms of the muramic acid moiety. It is tempting to speculate that conformational changes in muramic acid resulting from enzymatic regulation in vivo at the C_6 site serve to regulate SWS-promoting activity of MPs.

Experiment No. 4: Effects of MDP on Sleep, Body Temperature, and Plasma Copper

The present results clearly indicate that a central nervous system mechanism is involved in the increase in plasma Cu levels observed 27-28 hours after MDP administration since ICV administration of this agent induced this effect, whereas its IV injection at the same dose had no such effect. This increase in plasma Cu levels is probably an acute-phase response. Previously, MDP, given ICV, was shown to have the capacity to induce many responses characteristic of the acute-phase response, including fever (97). That enhanced SWS and reduced REM sleep also were induced by MDP given ICV therefore suggests that these responses may be additional facets of the acute-phase reaction. Indeed, we previously have postulated that the somnogenic effects of MDP and other immune response modifiers (e.g., interleukin-1 [66]) may be integral parts of the host defense syndrome (59).

Bacterial cell wall products, including several MPs, induce the synthesis and release of interleukin-1 in the host. It is possible that this endogenous substance mediates some or all of the effects of MPs. It has been shown previously that interleukin-1 (66) and certain substances which induce it, e.g., lipid A (61) and tumor necrosis factor (59), similarly induce the syndrome of enhanced SWS and fever. Interleukin-1 has also been characterized as the primary mediator of the acute phase response (17, 53, 70, 115). It has been shown that injection of interleukin-1 into the preoptic-anterior hypothalamus results in increased plasma Cu levels (13). Moreover, interleukin-1 has been described as an astrocyte product (35). Taken together, these findings suggest, therefore, that the effects after ICV administration which we report

here may be mediated, in part, via the central synthesis and release of interleukin-1.

Plasma Cu levels, fever, SWS, and REM sleep responses elicited by MDP may be regulated by independent central nervous system mechanisms. Thus, much previous evidence indicates that these responses were separable. Certain MPs induce fever without affecting sleep; other MPs enhance SWS without inhibiting REM sleep, while yet another inhibits REM sleep but does not affect SWS (64). Fever induced by MDP is attenuated by antipyretics without affecting SWS responses (63). MDP given to neonatal rabbits enhances quiet sleep, the precursor to SWS, without inducing fever (28), while endotoxin induces the acute-phase response without causing fever in neonatal guinea pigs (12). Prostaglandin E_2 injection into the preoptic-anterior hypothalamus induces fever without causing a rise in plasma Cu levels (14), while cyclooxygenase inhibitors block the febrile response to pyrogens but not the associated changes in glycoprotein plasma levels (13). Riveau et al. (97) elicited fever in some rabbits with MDP ICV administration without inducing a rise in plasma Cu. The double dissociation was demonstrated in the latter study by means of MDP derivatives which were not pyrogenic and yet induced a rise in plasma Cu. In addition, changes in peripheral or central temperatures (12) do not per se induce acute-phase resposes, attesting to the independence of these variables. Further, in the present study, we noted that a dissociation of responses occurred in seven animals. For example, after the low dose of MDP, one animal failed to show increased plasma Cu levels (89 μg/dl), but exhibited elevated SWS (62%) and body temperature (40.8°C) and reduced REM sleep (1.7%). In another animal, the respective values after the low dose of MDP were 201 μg/dl, 44%, 40.6°C, and 3.7%. Another case illustrates the independence of Cu re-

sponses from fever responses: in response to 15 nmol of MDP ICV, plasma Cu was 159 μg/dl; % SWS, 64%; body temperature, 39.5°C; and % REM sleep, 0.2%. Such dissociations do not lend themselves, however, to a simple interpretation in terms of MDP induction of central interleukin-1 release. Perhaps MDP and, by extension, interleukin-1, have differential effects on the biochemical mechanisms of the acute-phase response of sleep and fever (97).

Regardless of the question of separate mechanisms mediating different aspects of the acute-phase response, the simultaneous appearance of these responses (sleep, fever, and plasma Cu) after sufficiently high doses of MDP simulates a phenomenology common to infectious disease. Thus, it may provide an approach to the study of the possible adaptive significance of co-variation of these responses during the acute-phase response as well as of the neural mechanisms which coordinate these various responses.

Experiment No. 5: Effects of rTNF and rIL1 on Rabbit Sleep

The current results clearly indicate that rTNF and rIL1 have the capacity to enhance SWS. Previously, it was reported that rTNF enhances macrophage IL1 production and release (32); thus, it is possible that the somnogenic actions of rTNF are mediated through a step involving IL1 production. However, the time course of rTNF-induced effects on T_{br} and sleep suggest that TNF is intrinsically somnogenic. After ICV injections of rTNF, excess SWS was observed during the first hour after injection. Excess SWS is not observed during the first hour after injection of other somnogenic substances that enhance IL1 production, e.g., muramyl peptides (63). The biphasic fevers observed after rTNF injections suggest that perhaps the late-occurring rTNF-induced

excess SWS (i.e., 3-4 h after injection) was due to newly synthesized and released IL1.

The time courses of febrile responses induced by rIL1 and rTNF are substantially different from each other, although sleep responses induced by these substances are similar. In addition, the time courses of rTNF- and rIL1-induced fevers are different from their respective effects on sleep. This suggests that sleep responses are not secondary to fever responses. Other evidence supporting this conclusion was presented above. Nevertheless, it is well known that sleep is usually not accompanied by fever. The multiple actions of exogenous rIL1 and rTNF probably result from the rather unphysiological manner by which they were delivered. One would anticipate that if either one were involved in regulation of normal sleep, they would be delivered to effector brain sites by precise mechanisms in conjunction with a carefully orchestrated delivery of other neuromodulators, thereby increasing the specificity of response.

The rIL1 and the rTNF samples used in this study contained small amounts of endotoxin. Previously, it was shown that endotoxin itself is somnogenic (61); thus, it is possible that the sleep responses reported here are due to endotoxin rather than rIL1 or rTNF. However, certain characteristics of endotoxin-induced sleep and fever responses are substantially different from those reported here. For example, following ICV injection of lipid A, the active component of endotoxin, there is a 2-3 hour delay before effects on sleep are observed. In contrast, somnogenic doses of both rTNF and rIL1 enhanced SWS during the first hour after ICV injection. Following ICV injections of lipid A, REM sleep is not inhibited, whereas both rIL1 and rTNF

inhibited REM sleep. In addition, after ICV administration of rTNF, febrile responses are biphasic; and after IV injections of rIL1, fevers are monophasic; endotoxin induces monophasic fevers after ICV injections and biphasic fevers after IV injections (61).

The effects of rIL1 are similar to those induced by native IL1. Both native human IL1 (66) and human rIL1 enhance rabbit SWS, inhibit REM sleep, enhance EEG slow wave amplitudes, and induce monophasic fevers after ICV or IV injection. Further, the time courses of sleep effects after either ICV or IV injection of native IL1 or rIL1 are similar. Thus, with regard to fever and sleep activities, rIL1 is similar to native IL1. However, some activities of native IL1 have not been confirmed using rIL1, e.g., muscle degradation (32). These differences in activities may be due to structural differences between rIL1 and native IL1; e.g., the bacterial products are not glycosylated. Regardless of these structural differences, present data suggest that they are not important for somnogenic activity.

GENERAL DISCUSSION

TNF, IL1, and IFN (59) are somnogenic. In addition, several other substances have been described as putative sleep factors. These include PGD_2 (46), muramyl peptides (59, 60), lipid A/endotoxin (61), and vasoactive intestinal polypeptide (VIP) (95). It is, therefore, important to consider whether these substances may enhance sleep via a common biochemical-physiological mechanism and whether it is reasonable for more than one sleep factor to exist. Many of the substances mentioned above can affect each other's metabolism. A few examples illustrate: 1) TNF (32), muramyl peptides (73), and

lipid A (31) can enhance IL1 production; 2) IL1 can alter IFN metabolism (31); 3) TNF (32), IL1, muramyl peptides, endotoxin, and IFN can alter arachidonic acid metabolism (31), thus possibly altering PGD_2 production; and 4) PGD_2 can enhance release of VIP (104). Further, substances such as poly I:C, which are inducers of IFN (106), can also enhance sleep. These as well as other findings (reviewed in 59) suggest that the above-mentioned putative sleep factors may, in part, elicit their effects on sleep through a common mechanism. However, there is also evidence suggesting that these substances do not always work in concert with each other and may, therefore, independently enhance sleep. For example, poly I:C can enhance SWS, presumably through its ability to enhance IFN production (Endsley, unpublished). Poly I:C does not, however, affect one facet of the acute phase response, plasma Cu levels. If poly I:C had produced sleep through a step involving IL1, this result would be unlikely since IL1 elevates plasma Cu levels (13). Thus, these results suggest that there is more than one endogenous somnogen. Similar arguments have been made for the existence of multiple endogenous pyrogens (32).

There is also evidence for the existence of endogenous REM sleep factors (2), suggesting, therefore, that there are different sleep factors for different types of sleep. Further, it is reasonable to suppose that independent chemical messengers mediate the altered quantity and intensity (quality) of sleep that follow changes in either systemic or neuronal environments. It is also likely that various sets of neurons, each interacting with different sets of neuromodulators, are involved in sleep induction and maintenance. Moreover, it seems likely that within the CNS both glia and neurons are involved in sleep; IL1 (35) and IFN (72) are glial cell products. Thus, it is possible that substances that induce production of IFN and/or IL1, e.g., muramyl

peptides, may, in the CNS, first interact with astrocytes and/or microglia. In turn, these cells may release IFN and/or IL1, which in turn may affect sleep through interactions with various sets of neurons. Thus, if this scenario is correct, there would be at least two soluble endogenous substances capable of enhancing sleep; hence, two sleep factors. However sleep factors are defined, it is currently clear that more than one endogenous factor has the ability to enhance sleep.

Another context in which the effects of MPs have been discussed (59, 68) is the relation between the immune system and sleep. Our hypothesis is that one of the functions of sleep is to optimize conditions for and promote the function of the immune system. Thus, during infectious disease, the feeling of increased sleepiness which often occurs is interpreted as recruitment of sleep into the effort to combat disease. Similar mechanisms may also be evoked during prolonged wakefulness and during normal daily activity. In support of this hypothesis, we have shown that numerous substances known for their ability to stimulate certain aspects of the immune response, including MDP, also are somnogenic (reviewed in 59, 68). Further evidence suggesting such a relationship is the finding that narcolepsy is associated with the major histocompatibility class II marker, HLA-DR2 (called Ia in mouse); all narcoleptics possess this cell surface antigen, thus suggesting that narcolepsy is an autoimmune disease (9). MDP induces expression of Ia in glia. Although the relationships between the immune response and sleep remain to be clarified, it may be that sleep is involved in recuperative processes, whether it is recovery from a day's activity or a disease state.

Regardless of results from future experiments which will shed light on the above mentioned speculations, it is currently clear that certain MPs have the capacity to enhance SWS. Further knowledge of how they do so and of structural requirements for somnogenic activity will greatly enhance our ability to make an informed decision concerning the development of MPs as hypnotic agents.

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Table 1. Effects of MDP and Ambient Temperature on Rabbit Sleep and Brain Temperature

Group	Treatment	T_a (°C)	% SWS[a]	% REM[a]	T_{br}[b]
A1	No infusion	21°	37 ± 3	7 ± 3	39.3 ± 0.2
A2	Artificial CSF	21°	47 ± 3	10 ± 3	39.4 ± 0.1
A3	125 pmol MDP	21°	58 ± 4[c]	4 ± 3[c]	40.7 ± 0.2[c]
A4	125 pmol MDP DD	21°	44 ± 3	8 ± 2	39.4 ± 0.1
B1	No infusion	27°	55 ± 3[d]	6 ± 0.5	39.1 ± 0.2
B2	Artificial CSF	27°	57 ± 2	8 ± 1	39.3 ± 0.1
B3	125 pmol MDP	27°	67 ± 3[c]	4 ± 2[c]	40.3 ± 0.2[c]

a) Values are mean ± SEM for 6-h recording periods.

b) T_{br} are values determined 4 h after injections.

c) Significantly different from no infusion and artificial CSF infusion control groups.

d) Significantly different from A1 group.

Table 2. Effects of Various Treatments on Frequency of Sleep Episodes and Their Duration

I. Distribution of SWS

SWS Episode Duration[a]	A1[b] NI[21]	A2 CSF[21]	A3 MDP[21]	A4 MDP-DD[21]	B1 NI[27]	B2 CSF[27]	B3 MDP[27]
0-1	167	173	181	242	148	116	98
1-2	107	120	128	108	124	111	124
2-3	57	69	64	56	65	53	68
3-4	24	36	43	28	47	42	58
4-5	23	16	31	18	27	23	24
5-6	12	11	14	12	19	17	20
6-7	3	15	9	5	14	15	19
>8	12	10	22	15	18	17	29
Total No. of episodes[c]	405	450	492	484	462	394	440

II. Distribution of REM Sleep

REM Episode Duration[a]	A1[b] NI[21]	A2 CSF[21]	A3 MDP[21]	A4 MDP-DD[21]	B1 NI[27]	B2 CSF[27]	B3 MDP[27]
0-1	47	48	32	40	33	51	39
1-2	46	47	22	38	35	36	29
2-3	11	16	2	10	11	12	3
3-4	3	4	5	3	3	0	2
4-5	0	4	2	4	2	0	0
5-6	3	0	0	1	1	0	0
6-7	0	0	0	1	0	0	0
>8	0	0	0	0	0	0	0
Total No. of episodes[c]	110	121	63	97	85	102	73

(a) Values are in min.

(b) Group numbers A1, etc. refer to experimental group numbers described in Methods.

(c) Values shown are the total number of episodes that occurred in all 5 experimental animals under each condition.

Table 3. Effects of Intravenous Injections of Muramyl Dipeptide on Rabbit Sleep and Brain Temperatures during Dark and Light Phases

Group No.	MDP Dose (IV)	Phase	N[a]	% SWS[b]		% REM Sleep[b]		T_{br}[c]	
				Control	Experiment	Control	Experiment	Control	Experiment
1	5 μg/kg	Light	8	55 ± 2	59 ± 3	9.7 ± 1.4	6.1 ± 1.4	39.2 ± 0.1	40.0 ± 0.2[d]
2	5 μg/kg	Dark	8	45 ± 3	49 ± 4	5.5 ± 0.9[e]	7.0 ± 1.5	39.5 ± 0.1	39.2 ± 0.1[f]
3	25 μg/kg	Light	8	49 ± 2	59 ± 4[d]	11.0 ± 1.1	7.8 ± 1.7	39.2 ± 0.1	40.0 ± 0.3[d]
4	25 μg/kg	Dark	8	45 ± 3	50 ± 3	5.5 ± 0.9[e]	4.1 ± 0.5[f]	39.5 ± 0.1	39.6 ± 0.3

a) Groups 2, 3, and 4 were composed of same animals; 4 of these were also part of Group 1.

b) 6 h average ± SEM.

c) 4 h after injection.

d) Significantly different from corresponding control value.

e) Significantly different from control value obtained during light.

f) Significantly different from experimental value obtained during light.

TABLE 4: Effects of amphetamine and amphetamine plus muramyl dipeptide on SWS episode lengths, SWS episode number and brain temperature.

		Number of Episodes		Duration of Episodes		Temperatures	
Day of Assay	Substance Injected	min 60-180*	min 240-360*	min 60-180*	min 240-360*	min 120*	min 300*
1	Saline	31 ± 2	24 ± 1	1.9 ± 0.2	2.0 ± 0.1	39.2 ± 0.1	39.1 ± 0.2
2	MDP + Amphetamine	51 ± 7†§	37 ± 4§	1.3 ± 0.2	2.2 ± 0.2	40.1 ± 0.2§	40.5 ± 0.3†§
3	Saline	29 ± 4	28 ± 2	2.0 ± 0.3	2.1 ± 0.1	39.0 ± 0.1	39.0 ± 0.1
11	Saline	26 ± 3	25 ± 2	2.0 ± 0.2	2.0 ± 0.1	39.4 ± 0.1	39.4 ± 0.1
12	Amphetamine	21 ± 4	30 ± 5	1.2 ± 0.3	1.9 ± 0.3	39.6 ± 0.1	39.7 ± 0.1
26	Saline	29 ± 2	28 ± 4	2.1 ± 0.3	2.1 ± 0.3	39.1 ± 0.1	39.3 ± 0.1
27	Amphetamine + MDP	48 ± 8†§	35 ± 3	1.8 ± 0.4	2.7 ± 0.3	40.5 ± 0.5§	40.9 ± 0.4†§
28	Saline	34 ± 7	37 ± 9	1.6 ± 0.3	2.0 ± 0.4	40.3 ± 0.3§	40.3 ± 0.3§

*Min after beginning of experiments; these time periods were chosen because maximal effects of amphetamine were evident during the 60-180 min period and no effects were observable by 240-360 min.

†Significantly different from amphetamine alone treatment

§Significantly different from preceding saline values

Table 5. Effects of Several Bacterially Derived Muramyl Peptides on Rabbit Sleep

Structure	MW	Dose pmol	Effect on Temp	SWS	REM
NAG-(6,OAc)NAM-Ala-γGlu-A_{2pm}-Ala	981	1	0*	0*	0*
		10	+	+	0
NAG-NAM-ALa-γGlu-A_{2pm}-Ala(NH_2)	938	10	0	0	0
NAG-(1,6-anhydro)NAM-Ala-γGlu-A_{2pm}-Ala(NH_2)	920	10	0	0	0
NAG-NAM-Ala-γGlu-A_{2pm}-Ala	1,860	10	0	0	0
		100	0	0	0
NAG-NAM-Ala-γClu-A_{2pm}-Ala					
NAG-NAM-Ala-γGlu-A_{2pm}-Ala-Ala	1,931	10	0	0	0
		100	0	0	-
NAG-NAM-Ala-γGlu-A_{2pm}-Ala					
NAG-(1,6-anhydro)NAM-Ala-γGlu-A_{2pm}-Ala	1,842	10	+	0	0
		100	+	+	0
NAG-NAM-Ala-γGlu-A_{2pm}-Ala					

*0 = no effect; + = enhanced; - = decreased

Table 6. Effects of MDP on sleep, colonic temperature, and plasma Cu levels in rabbits.

Group	Dose	N	Cu μg/dl[a]	% SWS[b]	% REM sleep[c]	Colonic Temp.[d]
Naive	----	12	105 ± 11	N.D.[e]	N.D.	N.D.
Operated	----	6	101 ± 3	49 ± 3	9.9 ± 1.9	39.0 ± 0.1
PFS, ICV	25 μl PFS	8	83 ± 2[g]	50 ± 3	4.5 ± 0.9[g]	39.1 ± 0.1
MDP, ICV	150 pmol in 25 μl PFS	11	128 ± 10[fg]	62 ± 4[fg]	1.6 ± 0.4[fg]	40.7 ± 0.2[fg]
MDP, ICV	15 nmol in 25 μl PFS	7	168 ± 10[fg]	75 ± 3[fg]	0.1 ± 0.1[fg]	40.9 ± 0.3[fg]
MDP, IV	15 nmol in 0.5 ml PFS	6	87 ± 9	N.D.	N.D.	39.2 ± 0.1

[a]Plasma copper values (means ± SEM) determined 27-28 h after injection.

[b]% of time (means ± SEM) occupied by SWS during hours 0-6 after injection.

[c]% of time (means ± SEM) occupied by REM sleep during hours 0-6 after injection.

[d]Colonic temperature (means ± SEM) after 6 h of recording, or 3 h after IV injections, in °C.

[e]Not determined.

[f]Significantly different from PFS group.

[g]Significantly different from operated group.

Table 7. Effects of rTNF on rabbit sleep, EEG delta wave voltages, and brain temperature.

Route of Admin.	Dose	N	% SWS[a]		% REM[a]		Maximum delta wave amplitude, μV[b]		Average delta wave voltage[c]		Brain temperature[d]	
			Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
ICV	0.5 μg	6	50 ± 2	54 ± 2	7.7 ± 0.9	6.7 ± 0.8	161 ± 13	174 ± 14*[e]	84 ± 5	87 ± 6	39.2 ± 0.1	40.0 ± 0.1*
ICV	5.0 μg	4	51 ± 3	68 ± 2*	6.5 ± 0.6	1.8 ± 0.5*	148 ± 14	173 ± 24*	88 ± 6	108 ± 11*	39.2 ± 0.1	41.0 ± 0.2*
IV	1 μg/kg	6	55 ± 3	54 ± 2	7.4 ± 0.9	8.6 ± 1.6	157 ± 20	168 ± 27	82 ± 4	84 ± 6	39.1 ± 0.1	38.9 ± 0.1
IV	10 μg/kg	6	56 ± 3	69 ± 2*	7.4 ± 0.7	4.3 ± 0.6*	140 ± 15	166 ± 15*	83 ± 6	97 ± 7*	39.0 ± 0.1	40.5 ± 0.1*

[a]Values are means ± SEM for 6-h postinjection period.

[b]Values are means ± SEM of 12 maximum values of average delta wave voltages during bouts of slow wave sleep.

[c]Values are means ± SEM of average delta wave voltages for 6-h postinjection period.

[d]Values are means ± SEM of brain temperatures 3 h after injection.

[e]*indicates significantly different from control; paired t-tests were used; $p < 0.05$.

Table 8. Effects of rIL1 on rabbit sleep and brain temperature.

Lot No.	Route of Admin.	Dose	N	% SWS[a]		% REM[a]		Brain temperature[b]	
				Control	Exper.	Control	Exper.	Control	Exper.
1	ICV	50-100 ng	6	52 ± 3	50 ± 2	8.4 ± 0.7	7.9 ± 0.9	39.3 ± 0.1	39.3 ± 0.1
	ICV	200 ng	4	50 ± 1	57 ± 5	9.5 ± 1.8	7.9 ± 1.8	39.2 ± 0.1	40.0 ± 0.3*
	ICV	500 ng	4	55 ± 3	76 ± 4*[c]	7.3 ± 1.4	1.3 ± 0.5*	39.0 ± 0.1	41.8 ± 0.2*
	IV	1 μg/kg	4	48 ± 1	49 ± 3	4.8 ± 1.3	5.0 ± 1.2	39.3 ± 0.1	39.4 ± 0.1
	IV	10 μg/kg	5	52 ± 3	60 ± 4*	9.3 ± 1.7	4.8 ± 1.3*	39.3 ± 0.1	39.4 ± 0.3
2	ICV	50 ng	4	48 ± 3	66 ± 5*	1.7 ± 0.4	0.4 ± 0.3*	39.4 ± .5	40.5 ± 0.4*
	ICV	200 ng	4	58 ± 2	84 ± 3*	6.4 ± 0.6	0*	39.2 ± 0.1	41.8 ± 0.1*
	ICV	5,000 ng	4	56 ± 4	86 ± 2*	3.4 ± 1.0	0*	39.2 ± 0.2	40.3 ± 0.7[d]

[a]Values are means ± SEM for 6-h postinfusion period.

[b]Values are means ± SEM for brain temperature 3 h after infusion.

[c]*indicates significantly different from control; paired t-tests were used; $p < 0.05$.

[d]One of the animals was hypothermic after injection of this dose; another of the animals died three days after injection of this dose.

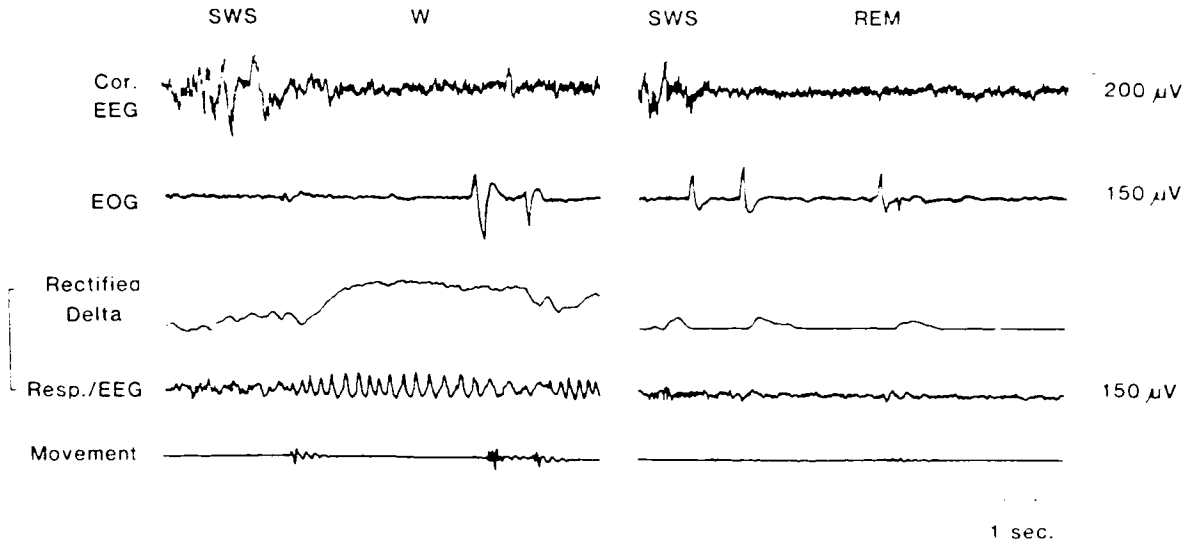

Figure 1: Relation of eye movements detected in the electro-oculogram (EOG) to cortical electroencephalogram (EEG), body movement, and nasal bone EEG (Resp/EEG). During transition from slow-wave sleep (SWS) to waking (W), nasal-bone delta-EEG (0.5-3.5 Hz) activity (rectified delta) of relatively high amplitude appears and is maintained during W immobility. The unfiltered nasal bone EEG (Resp/EEG) shows changes in amplitude which are associated with respiration during W. During transition from SWS to rapid eye movement (REM) sleep, Resp/EEG remains low in amplitude and fast. It is also noted that phasic eye movements in REM sleep can appear in the absence of body movement.

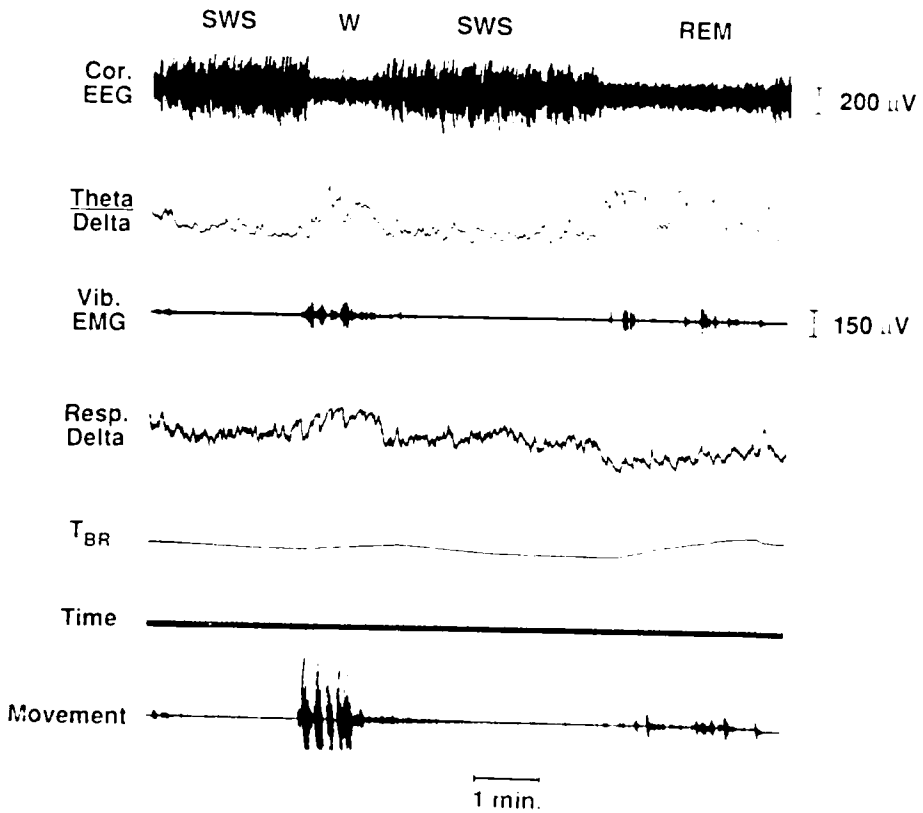

Figure 2: Definition of three stages of vigilance in rabbits. Waking (W) is defined by large body movements, moderate rise in brain temperature, presence of nasal bone delta EEG associated with respiration, variable but continuous bursts in facial musculature activity (vibrissae muscle electromyogram-EMG), cortical EEG of relatively low amplitude and with variable but relatively high theta (4-7 Hz) to delta ratio. Slow-wave sleep (SWS) is defined by relatively large EEG amplitudes of higher delta and lower theta activities, reduced motor activities (EMG and body movement), decreasing brain temperature and nasal bone EEG amplitude. Rapid eye movement (REM) sleep is defined by low amplitude EEG with steady reduction in delta and rise in theta activities (high theta/delta ratios), further reduced nasal bone delta-EEG activity, steep rise in brain temperature and occasional phasic events represented in this figure in vibrissae EMG bursts.

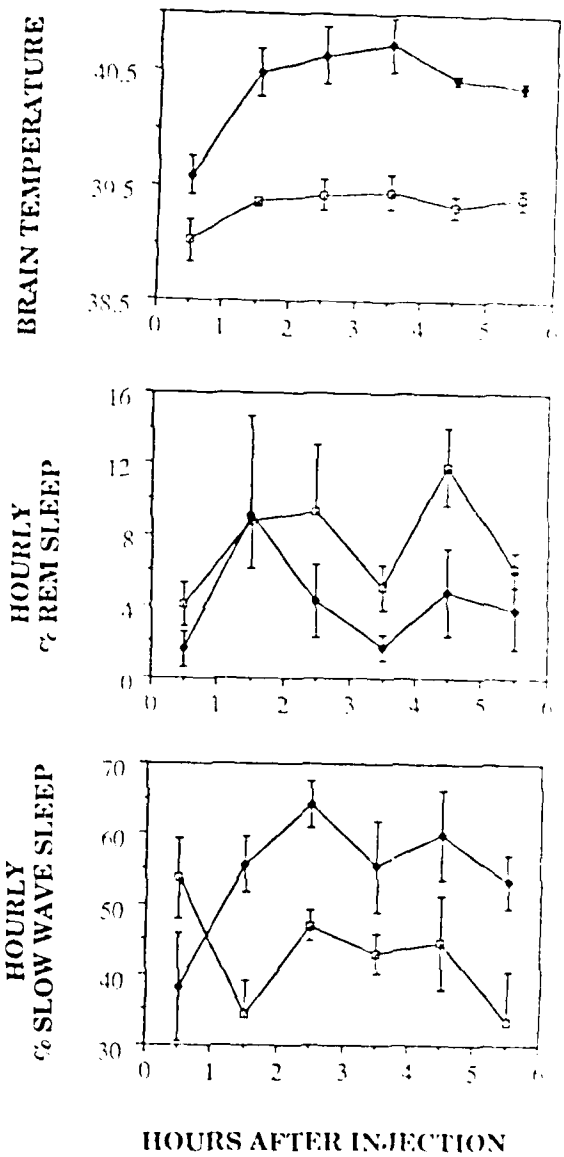

Figure 3: Effects of MDP and MDP-DD administered intracerebroventricularly (125 pmol/rabbit) during light hours at 21°C ambient temperature on rabbit sleep and brain temperature. MDP ( ) significantly reduced % SWS during the first hour. This was followed by elevated % SWS for the next 5 hrs compared to MDP-DD ( ). % REM was reduced by MDP whereas MDP-DD did not change % REM compared to no-infusion and artificial CSF controls (Table 1). MDP induced fever while MDP-DD did not.

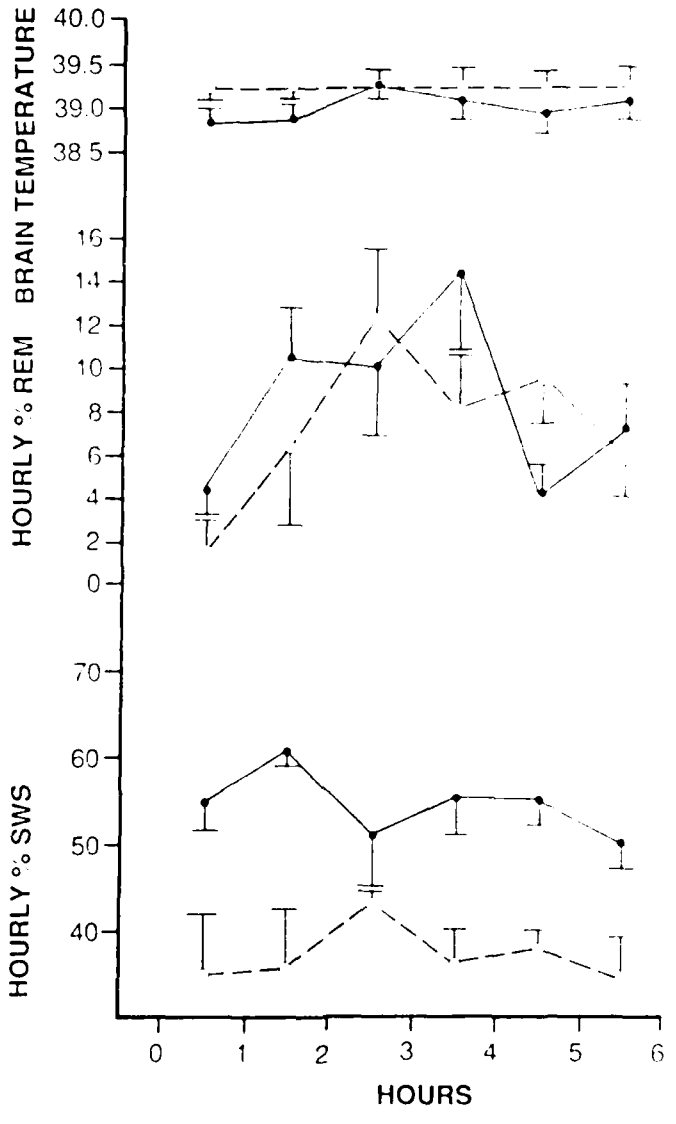

Figure 4: Effects of acute elevation of ambient temperature (T_a) from 21°C (o----o) to 27°C (o——o) on rabbit sleep and brain temperature. Neither group of animals (A1 and B1; see Methods) received infusion. The major effect of increasing T_a to 27°C was to increase duration of slow wave sleep during the entire 6-hour recording period; rapid eye movement was not significantly affected. Elevated T_a was also associated with lowered brain temperatures but this effect was not significant.

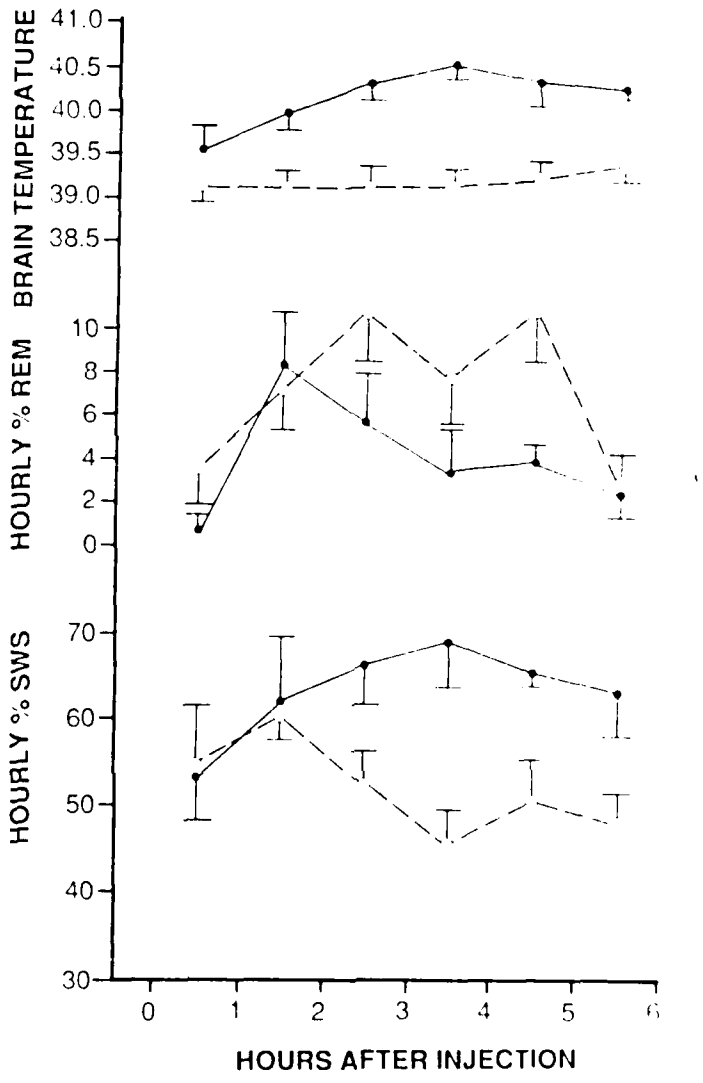

Figure 5: Effects of MDP at ambient temperature of 27°C MDP (o——o) (experimental group B3; see Methods) was given intracerebroventricularly (125 pmol) and its effects are compared to those of vehicle control (group B2, see Methods) (o----o). Injection of the control solution did not alter sleep or brain temperature compared to no infusion control taken at 27°C T_a (compare Figs. 4 and 5). However, injection of MDP at 27°C T_a further enhanced duration of slow wave sleep, increased brain temperature and suppressed rapid eye movement sleep. Thus, it is unlikely that MDP effects on sleep are mediated by environment thermoregulatory control of sleep.

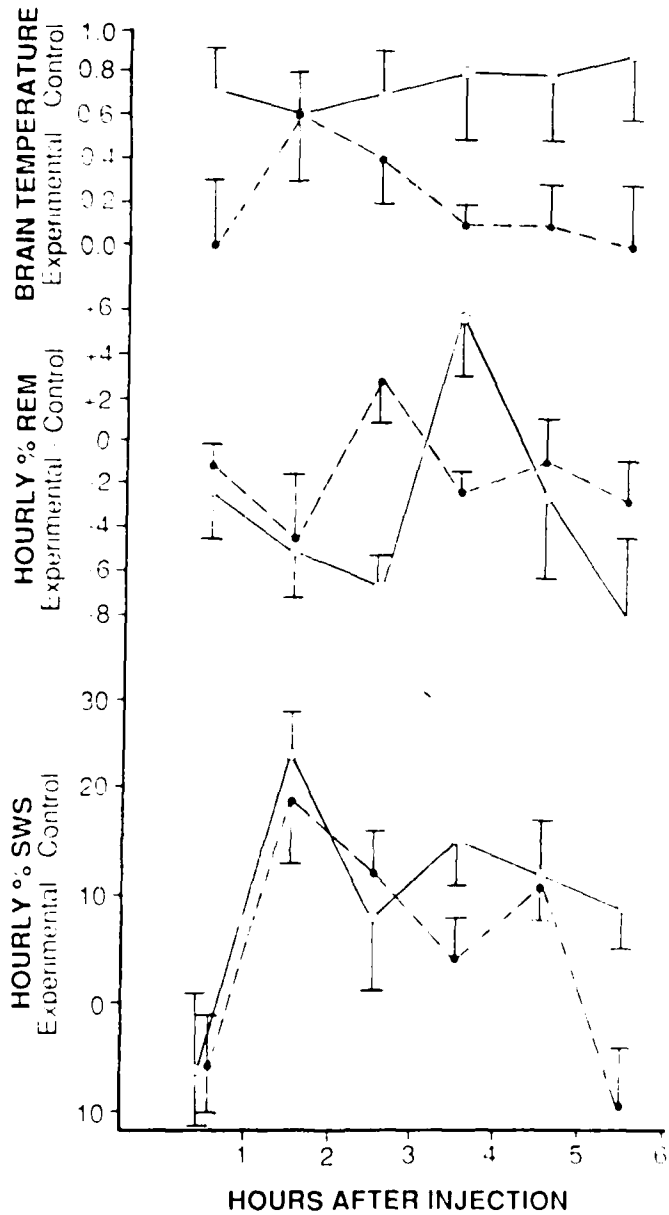

Figure 6: Time course of effects of MDP during dark (o----o) and light (o——o) periods after intravenous administration of 25 μg/kg. The effects of MDP on slow-wave sleep (SWS) were similar during both light and dark phases. During the dark phase, duration of REM sleep was reduced in control animals (see Table 3). MDP did not further reduce REM sleep during this time. However, during the light phase, MDP induced significant reductions in REM sleep. During the dark phase, the effects of MDP on brain temperature were much attenuated compared to those elicited during light hours.

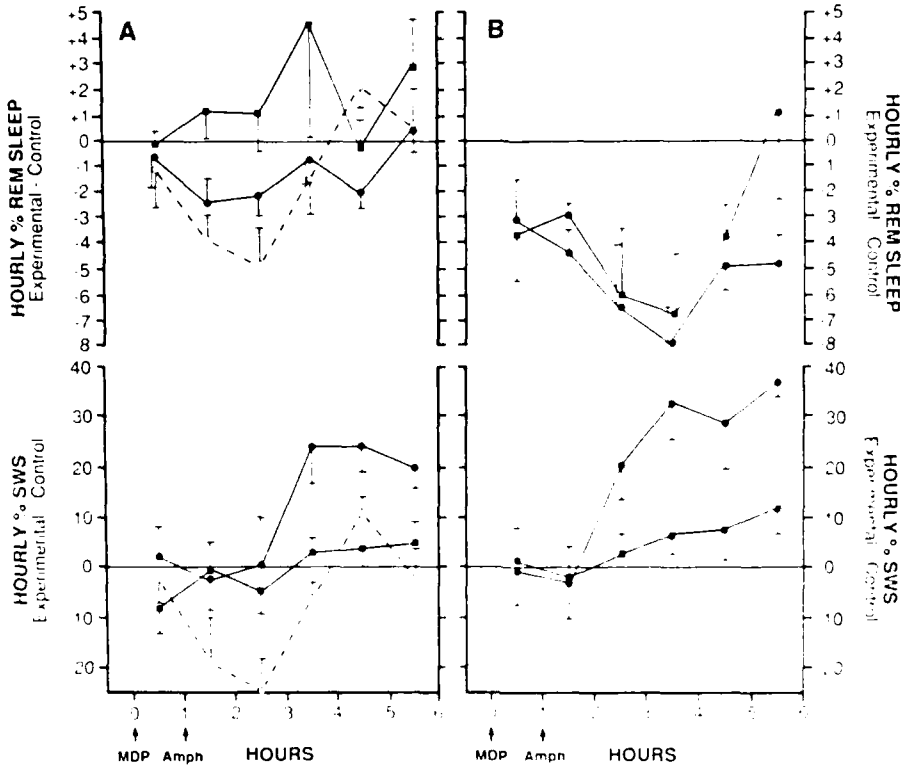

Figure 7: Effects of amphetamine, muramyl dipeptide (MDP), and control injections on rabbit slow-wave and rapid eye movement sleep. Results are expressed as the difference between experimental and control values. Values shown are as follows; see Methods for specific protocol. The times various injections were made are indicated on the abscissa. A, ( ) effects of 1 mg/kg IV amphetamine alone [values from day 1 subtracted from corresponding values obtained on day 12]; ( ) effects of 1 mg/kg IV amphetamine plus 150 pmol ICV MDP [day 2 - day 1]; ( ) effects of control injections [day 3 - day 1]. B, ( ) effects of 1 mg/kg IV amphetamine plus 12,500 pmol ICV MDP [day 27 - day 26]; ( ) effects of control injections [day 28 - day 26]. Amphetamine induced a transient reduction in SWS and REM sleep for about 2 hours. MDP reversed this amphetamine-induced insomnia. The effects of the higher dose of MDP (B) on REM sleep persisted the day after MDP injection; the higher dose of MDP also affected body temperatures the day after its injection. The effects of the low dose of MDP alone in a different set of animals are shown in Fig. 8.

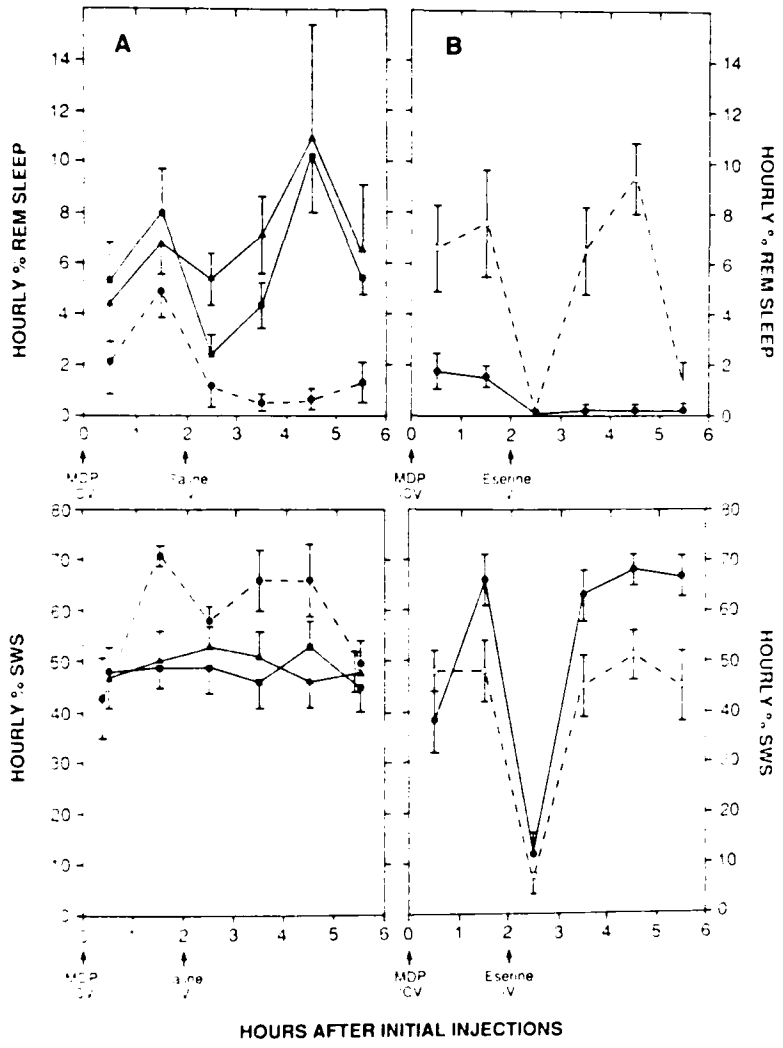

Figure 8: Effects of MDP and eserine on duration of rabbit sleep. Results are expressed as hourly percent spent in each state of sleep. A, ( ) effects of 125 pmol ICV MDP alone; ( ) effects of control injections before MDP treatment; ( ) effects of control injections after MDP treatment. (B) ( ) effects of 0.15 mg/kg IV eserine alone; ( ) effects of 125 pmol ICV MDP plus 0.15 mg/kg IV eserine. See Methods for specific protocol. Eserine induced insomnia that lasted about 1 hour; this effect was not reversed by MDP.

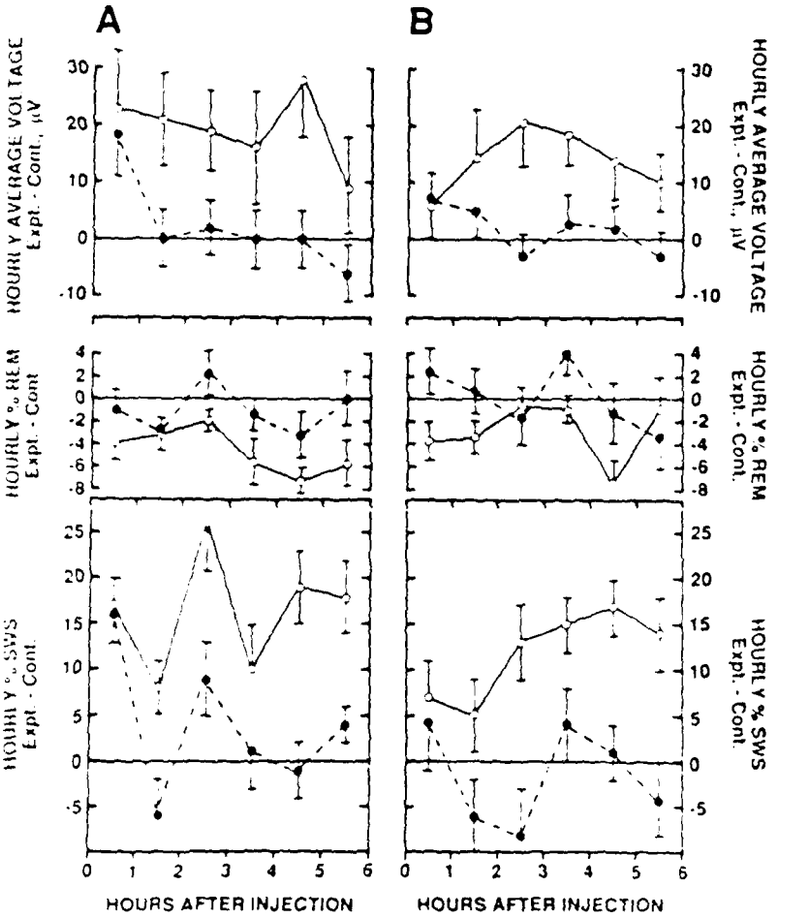

Figure 9: Effects of intracerebral ventricular (ICV) administration (A) compared with effects of intravenous (IV) administration (B) of rTNF on rabbit SWS, REM sleep, and EEG slow wave (0.5-4 Hz) voltages. A: open circles-solid lines, effects of 5.0 μg rTNF (n = 4); closed circles-broken lines, effects of 0.5 μg rTNF (n = 6). B: open circles-solid lines, effects of 10 μg/kg rTNF (n = 6); closed circles-broken lines, effects of 1 μg/kg rTNF (n = 6). Values shown are means ± SEM of individual experimental-control values. SWS and amplitude of EEG slow waves were significantly enhanced during the first h postinjection after both doses given ICV. After IV injection of rTNF, significant increases in SWS were not observed until the third h postinjection. REM sleep was inhibited after the high ICV and IV doses.

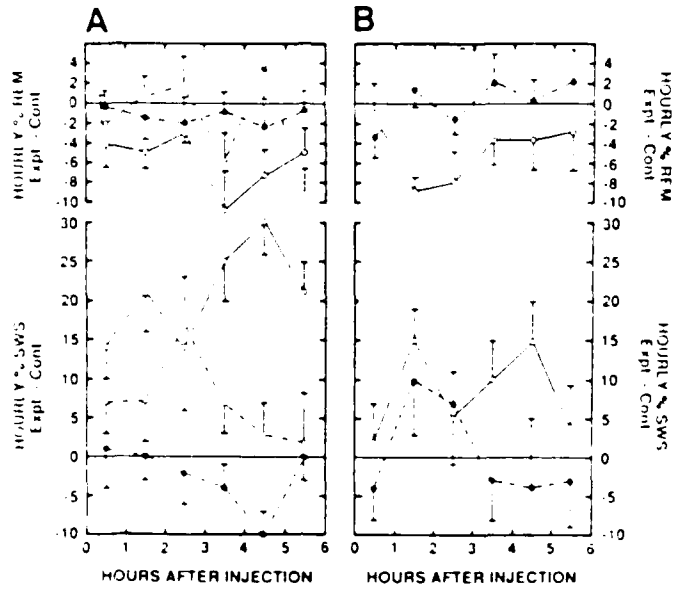

Figure 10: Time courses of effects of rIL1 on rabbit SWS and REM sleep after intracerebral ventricular (ICV) (A) and intravenous (IV) (B) administration. A: o——o, 500 ng (n = 4); Δ---Δ, 200 ng (n = 4); ●---●, 100 ng (n = 6) of lot No. 1 rIL1. B: o——o, 10 μg/kg (n = 5), and ●---●, 1.0 μg/kg (n = 4) of lot No. 1 rIL1. Values shown are means ± SEM of individual experimental-control values. After the higher doses of rIL1, SWS was enhanced and REM sleep was inhibited. Time courses of these sleep effects were similar to those observed after rTNF administration and were similar to those induced by native IL1 (67). Similar effects were observed after injection of rIL1, lot No. 2, although lot No. 2 was more potent.

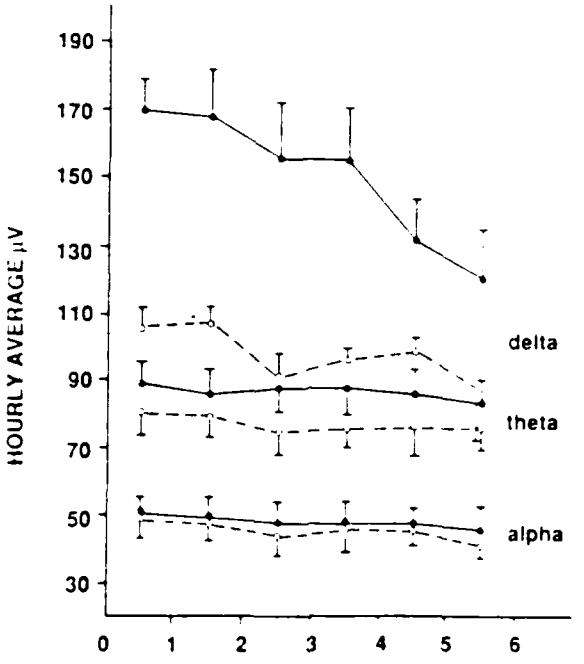

Figure 11: Effects of recombinant IL1 (rIL1) on hourly average voltages in various frequency bands. (●——●), effects following 200 ng rIL1 (lot No. 2) ICV (N=4); o---o, results from same rabbits without injection. Values for the beta frequency band were also determined; they are not shown because they were very close to those obtained in the alpha band. rIL1 greatly enhanced the average voltage in the delta frequency band. Average voltages in the other frequency bands were not affected to the same extent, although theta voltages after rIL1-treatment were significantly higher than those observed during control conditions.

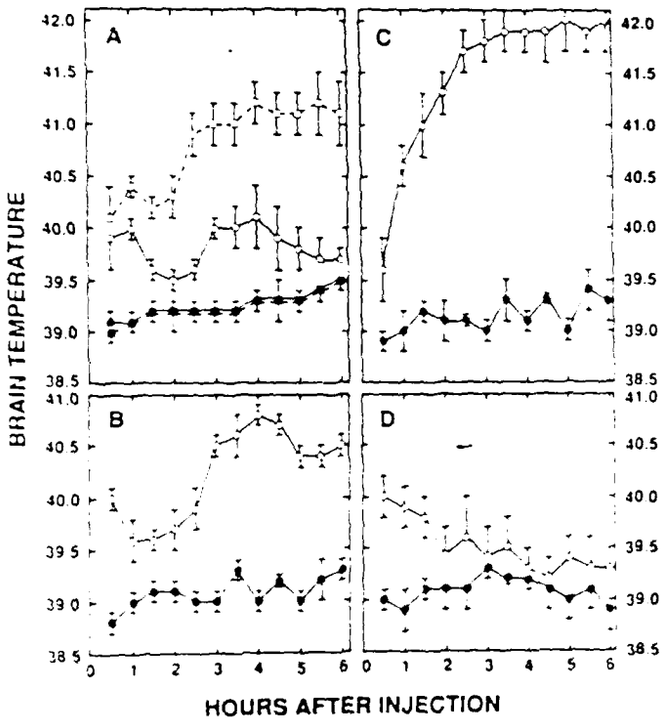

Figure 12: Time course of effects of somnogenic doses of rIL1 and rTNF on rabbit brain temperature. A: effects of rTNF after ICV injection of 0.5 μg (o——o) and 5.0 μg (□- - -□). B: effect of rTNF after IV injection of 10 μg/kg (o——o). C: effects of rIL1 after ICV injection of lot No. 1, 500 ng (o——o). D: effects of rIL1 after IV injections of lot No. 1, 10 μg/kg (o——o). In A, B, C, and D, control values from same rabbits are illustrated with closed circles (or squares). All somnogenic doses of rIL1 and rTNF induced fevers. However, the time courses of fevers were different from the time courses of rTNF- or rIL1-induced sleep effects. In addition, the time courses of rTNF-induced fever are substantially different from those induced by rIL1.

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